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SERUM DIAGNOSIS OF SYPHILIS
BY PRECIPITATION



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SERUM
DIAGNOSIS OF SYPHILIS
BY
PRECIPITATION

Governing Principles
Procedure and Clinical Application
of the
Kahn Precipitation Test

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To
LAFAYETTE B. MENDEL

AND

WILLIAM H. PARK

*In Whose Laboratories the
Author Learned Methods of Research*

PREFACE

This volume presents the experimental studies carried out by the author during the past three years on the precipitation phenomenon in syphilis and gives the development, standardization and clinical application of the author's test. It was believed that the publication of these studies under one cover would permit better correlation of the factors governing precipitation in syphilis and lead to a better understanding of the principles of the test than would the publication of separate papers.

A complete discussion of the procedures embraced by the author's precipitation system is given, although, with the exception of the "presumptive" procedure, outlines of technic have already been published. The part dealing with clinical application should be of especial interest to physicians and students of syphilology.

Acknowledgment is due Dr. Udo J. Wile and Dr. Harther L. Keim of the Department of Dermatology and Syphilology of the University of Michigan Medical School, whose interest in studying the clinical value of the test has been a source of encouragement to the author in his continued studies. Chapter XXIII presents a résumé of clinical studies on the Routine Test carried out with Dr. Keim, fully published elsewhere, and is included in this volume with his kind permission.

The writer desires to express his indebtedness to Dr. F. G. Novy of the University of Michigan, for reading the manuscript and making helpful suggestions. For reading parts of the manuscript, the writer is indebted to his former teacher, Dr. Wm. H. Park, and to Miss M. A. Wilson of the New York City Department of Health; also to Dr. Israel Weinstein, New York, N. Y., and to Dr. Geo. C. Stucky of the staff of Ford Hospital, Detroit.

The writer is particularly indebted to Dr. R. M. Olin, Michigan Commissioner of Health, and to Dr. C. C. Young, Director of the Bureau of Laboratories, whose coöperation and efficient administration have helped to make this work possible. The writer is also indebted to Miss Marjorie Delavan, Director of the Bureau of Education, for reading the manuscript.

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The service rendered by Miss Pearl L. Kendrick, assistant immunologist, has been invaluable and the writer takes this opportunity to express his gratitude.

R. L. KAHN

*Bureau of Laboratories
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Lansing, Michigan
August, 1924*

CONTENTS

PART I. INTRODUCTORY

CHAPTER I

INTRODUCTION.....	3
-------------------	---

CHAPTER II

PRECIPITATION TESTS PROPOSED BY DIFFERENT AUTHORS.....	7
--	---

PART II. GOVERNING PRINCIPLES OF PRECIPITATION AND THEIR APPLICATION TO AUTHOR'S TEST

CHAPTER III

OBSERVATIONS LEADING TO DEVELOPMENT OF TEST.....	19
Recognition of Importance of Concentration of Ingredients....	19
Early Recognized Requirements for a Practical Precipitation Test.....	25
Author's Early Precipitation Procedure.....	28
Elimination of Incubation.....	31
Author's Present Precipitation Method.....	32

CHAPTER IV

RÔLE OF CONCENTRATION.....	35
Delaying Effect of Salt Solution on Precipitation.....	35
Effect of Concentration upon Agglutination of Precipitate.....	39
Concentration vs. Dilution in Three Different Precipitation Tests.	40

CHAPTER V

INSTABILITY OF ANTIGEN DILUTION.....	42
An Antigen-Saline Precipitate Soluble in Serum.....	42
Minimum Amount of Saline in Antigen Dilution.....	44
Unstable Antigen Dilution Essential for Sensitiveness.....	46
Importance of Uniformity in Dilution of Antigen.....	49
Use of Saline in Standardizing Antigen Dilution.....	51
Effect of Age on Sensitiveness of Antigen Dilution.....	51

CHAPTER VI

QUANTITATIVE RELATION BETWEEN SERUM AND ANTIGEN.....	54
Quantitative Requirements for Complete Precipitation Reaction.....	54
The Serum-Antigen Amounts in Routine Tests.....	57
Direct Determination of Number of Reacting Substances in Syphilitic Sera.....	58
Absorption of Serum Reacting Substances with Antigen.....	63
Effect of Excessive Amounts of Serum.....	64
An Anomalous Reaction.....	64
Importance of Quantitative Measurements of Serum Substances in Syphilis.....	65

CHAPTER VII

FACTORS AFFECTING SENSITIVENESS AND UNIFORMITY OF ANTIGEN ..	68
Preliminary Extraction with Ether.....	68
Extraction with Alcohol.....	76
Cholesterinization.....	77

CHAPTER VIII

FACTORS AFFECTING SERUM.....	80
Unheated Versus Heated Sera.....	80
Period of Inactivation.....	82
Effect of Contamination and Age on Sera.....	86
Anticomplementary Sera.....	89

CHAPTER IX

SHAKING OR AGITATION.....	90
Effect of Shaking on Sensitiveness of Reactions.....	90

PART III. SPECIAL STUDIES ON AUTHOR'S TEST

CHAPTER X

COMPONENT OF ANTIGEN DILUTION RESPONSIBLE FOR SPECIFIC PRECIPITATION.....	99
---	----

CHAPTER XI

EFFECT OF INCUBATION ON TEST.....	104
-----------------------------------	-----

CHAPTER XII

EFFECT OF ACID AND ALKALI, ON TEST.....	109
---	-----

CHAPTER XIII

USE OF DIFFERENT ANTIGENS IN TEST.....	115
--	-----

CHAPTER XIV

AN ESPECIALLY SENSITIVE ANTIGEN.....	121
--------------------------------------	-----

PART IV. AUTHOR'S PRECIPITATION SYSTEM—
PROCEDURE

CHAPTER XV

GLASSWARE AND APPARATUS.....	129
------------------------------	-----

CHAPTER XVI

INGREDIENTS OF TEST.....	131
--------------------------	-----

CHAPTER XVII

TITRATION OF ANTIGEN FOR TEST WITH SERUM.....	136
---	-----

CHAPTER XVIII

ROUTINE TEST WITH SERUM.....	139
------------------------------	-----

CHAPTER XIX

QUANTITATIVE PROCEDURE WITH SERUM.....	148
--	-----

CHAPTER XX

PROCEDURE WITH SPINAL FLUID.....	153
----------------------------------	-----

CHAPTER XXI

PRESUMPTIVE PROCEDURE OF HIGH SENSITIVENESS.....	160
--	-----

PART V. CLINICAL APPLICATION

CHAPTER XXII

ONE HUNDRED THOUSAND EXAMINATIONS COMPARED WITH WASSER- MANN TEST.....	167
---	-----

CHAPTER XXIII

THE ROUTINE TEST IN THE DIAGNOSIS OF SYPHILIS.....	172
--	-----

CHAPTER XXIV

THE ROUTINE TEST IN CASES UNDERGOING TREATMENT.....	181
---	-----

CHAPTER XXV

THE QUANTITATIVE PROCEDURE IN CASES UNDERGOING TREATMENT.	185
---	-----

CHAPTER XXVI

APPLICATION OF SPINAL FLUID PROCEDURE.....	188
--	-----

CHAPTER XXVII

APPLICATION OF PRESUMPTIVE PROCEDURE.....	191
---	-----

PART VI. GENERAL CONSIDERATIONS

CHAPTER XXVIII

SUMMARY OF AUTHOR'S STUDIES—WITH CONSIDERATIONS OF SOME	
FURTHER INVESTIGATIONS.....	197
Concentration of Ingredients.....	198
Unstable Antigen Dilution.....	201
Relation between Serum and Antigen.....	205
Sensitiveness and Uniformity of Antigen.....	206
Studies on Serum.....	211
Agitation or Shaking.....	213
Increasing the Sensitiveness of Precipitation Reactions.....	213
Study of Individual Sera for the Presence of Minute Amounts	
of "Reagin".....	215
Clinical Investigations.....	216
SUMMARY CHART.....	facing 218
BIBLIOGRAPHY.....	219
INDEX.....	227

PART I
INTRODUCTORY

CHAPTER I

INTRODUCTION

Practically all alcoholic extracts of animal tissue, no matter how prepared, when properly mixed with syphilitic sera, will produce precipitates after sufficient incubation. This, it would appear, might render the evolvement of a precipitation test for syphilis comparatively easy. Yet, of the number of precipitation tests proposed, none have received universal application. Our studies indicate that we have been overlooking some fundamental factors which govern the precipitation phenomenon in syphilis. We consider the first factor in the construction of a precipitation test for syphilis to be concentration of the ingredients. Serum, antigen and normal salt solution must be so used as to assure as high a degree of concentration as the mechanism of the test permits. No precipitation test, in our opinion, can have permanent value unless this requirement has received major consideration in its evolvement. Experience gained with over 100,000 tests carried out in this laboratory leads us to believe that one of the main difficulties with most of the precipitation tests heretofore proposed is that the basis of their development is dilution rather than concentration.

One of the outstanding effects of concentration appears to be the immediate formation of precipitates. As will be shown later, concentration plays an important part in eliminating the necessity for incubating the serum-antigen mixtures in order to bring about precipitation. In the test proposed by the author the stronger sera show complete precipitation in about 15 seconds and the weaker sera in approximately 2 minutes after mixing serum with antigen

dilution. The practical value of immediate reactions is obvious: It facilitates repetition whenever there is the slightest suggestion of error; it does away with bacterial contamination which may result from incubation, particularly when associated with a high degree of dilution; it is of especial help in emergency cases, such as blood transfusion or surgical cases, where the rapidity of obtaining results is an important factor.

There are, however, still other effects of concentration which play an important part in a precipitation test for syphilis. The nature of the precipitate is influenced by the degree of concentration. Although precipitation implies some degree of agglutination or clumping of the particles, further agglutination apparently takes place as a distinct phenomenon and appears to be markedly affected by the degree of concentration. Due to this effect, a concentrated serum-antigen mixture forms, in positive sera, relatively heavier precipitates than a dilute mixture. This, in turn, renders the reading of final results comparatively easy. Finally, what is undoubtedly of the highest importance, clinical studies indicate that concentration has a direct effect on the sensitiveness and the specificity of a test.

Next in importance to concentration appears to be the instability of the antigen-saline mixture which is used with serum. The greater the instability of this mixture, the more marked the specific precipitation when employed with serum. This might be expected. The reacting substances in a stable antigen-saline mixture are not likely to combine as readily with the specific serum elements as the reacting substances of an unstable mixture. The antigen-saline mixture used in the author's test contains a precipitate, but this precipitate is so unstable that it readily dissolves in serum or salt solution.

Another important factor in the development of a practical precipitation test for syphilis is the employment of correct

serum-antigen proportions. Experiments indicate that, for complete precipitation in any given serum-antigen mixture, there must exist a proper quantitative relationship between the reacting substances in the serum and the reacting substances in the antigen. Sera from different conditions of syphilis give precipitation reactions only with definite amounts of antigen, and to vary these amounts may render positive reactions negative. An obvious solution to this problem is to use varying proportions of serum and antigen. More specifically, what seems desirable in a given test is the employment of several similar amounts of serum with such amounts of antigen as experiments indicate would be likely to meet the quantitative antigen demands of sera from different conditions of syphilis. The strongly positive sera, it is true, may give complete precipitation with all the various antigen amounts, but the weaker sera, instead of giving weak reactions in the usual sense of the term, are likely to give complete precipitation with at least one of the antigen amounts. This subject will be more fully discussed in the text. This, in our opinion, is clear: Only by meeting the different antigen requirements of various syphilitic sera can a test attain practical value in the realm of precipitation.

Assuming that the requirements of concentration, instability of antigen dilution and correct relation between serum and antigen have been met in a precipitation test, there are still other phases which must receive careful consideration. What should be the guiding basis for the preparation of antigen? Furthermore, how can one overcome the fluctuations inherent in alcoholic extractions of heart muscle—extractions which, although carried out similarly, may lead to the production of antigens showing considerable variation in lipoid content? Indeed, it is not unlikely that the varying results reported with most precipitation tests may be largely due to the fact that the

antigens prepared by different workers—although presumably according to the same directions—have lacked uniformity. Granting that this variable element in antigen preparation can not be entirely overcome, our studies indicate that this element may be reduced to such a degree that its influence on the test is practically negligible.

Then again, how should the antigen be titrated or standardized for the test? How is the test to be performed and how are the results to be interpreted? These and many other problems present themselves in the evolution of a precipitation test.

Although there have appeared during the past two years a number of reports from this laboratory on the author's precipitation test (1), these have been essentially outlines of the technic and preliminary in character. Before attempting to discuss the underlying principles of the precipitation phenomenon in syphilis, in so far as they are related to the author's test, it was felt necessary to first overcome as many difficulties inherent in the method as possible, to determine the practical value of the test on a comparatively large scale, and to establish its specificity for syphilis by extensive clinical studies.

It is not to be assumed that the test, as presented, is the "final word" in the serum diagnosis of syphilis by precipitation. No test which employs as an antigen alcoholic extracts of heart tissue can possibly represent the final word in the diagnosis of an infectious disease. On the other hand, the high degree of specificity and sensitiveness of the author's test combined with the simplicity of procedure may indeed not only render it a valuable method for the serum diagnosis of syphilis, but, what is no less important may actually open up new channels for serological research in syphilis and ultimately help solve the numerous problems in this field, particularly the mystery as to the relation of beef heart antigen to syphilis.

CHAPTER II

PRECIPITATION TESTS PROPOSED BY DIFFERENT AUTHORS

This résumé will be limited to precipitation reactions in which tissue extracts are employed as antigens. As is well known, numerous precipitation tests have been proposed in which substances other than tissue extracts are used, as for example the Klausner (2) reaction in which distilled water is used as an antigen and the Porges and Meier (3) reaction which employs lecithin suspensions.

Michaelis (4) reported a precipitation reaction for syphilis one year after Wassermann, Neisser and Bruck published the well known Wassermann test. He employed 0.2 cc. of serum, 0.2 cc. of a watery extract of syphilitic liver and 0.9 cc. of normal salt solution. He believed with the workers of that day that the reaction was specific, the serum containing the antibody and the extract, the specific antigen.

Three years later, Jacobstahl (5) published an ultramicroscopic precipitation test for syphilis. He employed 0.1 cc. of serum, 0.4 cc. of alcoholic extract of syphilitic liver (diluted 1:5) and 0.5 cc. salt solution. After incubation of the tests for 2 hours at 37°C. followed by 4 to 6 hours at room temperature, this worker was able to see in positive sera precipitation particles by means of the ultramicroscope. When the incubation period was extended from 24 to 48 hours, he observed a macroscopic precipitate in 50 to 75 per cent of cases.

The studies of Bruck and Hidaka (6) soon followed. They also employed alcoholic extracts of syphilitic liver. They showed that the Jacobstahl microscopic method was not practical and that a macroscopic precipitate could

be obtained by employing the following procedure: 0.8 cc. salt solution is added to 0.2 cc. of serum (active or inactive); 0.25 cc. of alcoholic liver extract is added to 1 cc. salt solution. The serum is mixed with the diluted extract, centrifuged for a short time, incubated for 1 hour and again centrifuged for 15 minutes. Precipitates are found in both positive and negative sera. Slight shaking causes the precipitates to go back into solution in the case of negative sera, but has no effect upon the precipitates formed in positive sera. If the serum-antigen mixture is incubated for 24 hours at 2° to 4°C. and then centrifuged, the precipitates are found considerably heavier in the case of the positive sera, while those in the negative sera go back into solution.

Hecht (7) in 1915 evolved an antigen to be employed both for the Wassermann test and for a precipitation procedure. He prepared the antigen mixture by adding 0.9 gram sodium chloride to 20 cc. of alcoholic extract of heart muscle, evaporating nearly to dryness, and subsequently adding 100 cc. distilled water to the lipoidal mass. Although this final solution was slightly turbid, it permitted light to pass through and did not form any precipitate on standing. For the test, 1 cc. of this lipoidal emulsion is mixed with 0.2 cc. of active or inactive serum and incubated for about 8 hours, after which time syphilitic serum shows a heavy precipitate while normal serum shows no change.

Meinicke (8) in 1917 reported his original water and salt solution precipitation methods for the diagnosis of syphilis. In his water method, 0.2 cc. of inactivated serum is mixed with 1.5 cc. alcoholic extract antigen diluted 1:12. The mixture is incubated for 1 hour at 37°C. and, after 2.5 cc. distilled water are added, it is incubated for 16 hours more. The optimum amounts of serum and antigen are determined by titration. Negative sera show distinct flocculation while positive ones show slight turbidity but no flocculi.

In the salt solution method, 0.2 cc. inactivated serum is mixed with 2 cc. of antigen-distilled water suspension of 1:8 dilution. The mixture is shaken and incubated for 16 hours at 37°C. All sera show the presence of a precipitate. Distilled water, 1 cc. to each tube, is added and the tubes are shaken until all show flocculi of similar size. To each tube is carefully added 1 cc. of 2.5 per cent salt solution without shaking. The tests are incubated for 1 hour, after which the precipitates in the negative sera are found to be dissolved, while the precipitates in the positive sera are unaffected.

One year after the publication of his first paper, Meinicke reported his Third Modification. This modification is supposed to be a combination of the water and salt solution methods and is carried out as follows: The antigen is prepared from dried and ground horse heart. Before extracting with alcohol, the heart muscle is shaken with ether (1:10) for 1 hour. After filtering off the ether the material is dried and then extracted for 1 day with 10 times its quantity of alcohol. The filtrate is ready for use after it has stood for several days. Before using, however, each antigen is titrated with 96 per cent alcohol and distilled water in order to determine to what extent the antigen is to be diluted with alcohol for correct tests. This titration is carried out as follows: Four tubes are set up. Tube 1 receives 0.4 cc. antigen and 0.1 cc. alcohol; tube 2, 0.3 cc. antigen and 0.2 cc. alcohol; tube 3, 0.2 cc. antigen and 0.3 cc. alcohol; and tube 4 receives 0.1 cc. antigen and 0.4 cc. alcohol. To each tube is then added 0.25 cc. distilled water and all tubes are permitted to stand 1 hour. After this period, 3.5 cc. distilled water are added to each tube and the one showing opalescence with total freedom from a precipitate contains the proper combination of alcohol and antigen for use in the tests. In most cases, 2 parts of antigen and 3 parts of alcohol represent the desired combination.

After mixing the proper amount of alcohol with the extract antigen as indicated by the above titration, a definite amount of this solution, depending on the number of tests to be run, is mixed with half the quantity of distilled water and permitted to stand for 1 hour. After this period, seven times the quantity of 2 per cent sodium chloride solution is added and the mixture is ready for use. (Thus, 5 cc. of antigen are mixed with 2.5 cc. of distilled water and after 1 hour 35 cc. of 2 per cent salt solution are added.) For a given test, 0.8 cc. of this antigen—distilled water—2 per cent salt solution mixture and 0.2 cc. of inactivated serum are employed. Readings are made by means of an agglutinoscope after 24 hours' incubation at 37°C. and are checked by another reading after 24 hours' further incubation at room temperature.

Under the term "Lipoidbindungreaktion," Meinicke attempts to show that he is dealing in his water method, salt method, and third modification, with a phenomenon similar to complement binding. In the latter case, the antigen and antibody unite and bring about the binding of complement, the indicator being some hemolytic system. In the lipid binding reaction, the specific antigenic substance of the extract unites with the specific antibody and binds the lipoids. The indicator is precipitation of globulins (globulin fällung) in the case of the third modification and solution of the globulins (globulinlösung) in the water and salt solution methods. The underlying factor in the lipid binding reaction appears to be the withdrawal of salt from the serum globulins by the lipoids. For a complete discussion of the mechanism of these precipitation tests, the reader is referred to Meinicke's original papers.

Sachs and Georgi (9) published their well known precipitation test in 1918. The antigen is prepared by extracting ground wet heart muscle with 5 times the quantity of alcohol for a day. The extract is permitted to stand

for several days in the icebox and refiltered to remove the sediment. Originally, Sachs and Georgi suggested adding 200 cc. of alcohol to 100 cc. alcoholic filtrate and cholesterinizing this by adding 13.5 cc. of 1 per cent alcoholic solution of cholesterin per 100 cc. diluted extract. Later, they suggested determining by titration the amounts of alcohol and cholesterin to add to the alcoholic extract. The following titration is usually employed (10):

The alcoholic extract is diluted

1. With equal parts of alcohol
2. With 2 parts of alcohol
3. With 3 parts of alcohol

To 10 cc. of each of these dilutions are added

1. 0.4 cc. of a 1 per cent cholesterin solution
2. 0.6 cc. of a 1 per cent cholesterin solution
3. 0.8 cc. of a 1 per cent cholesterin solution
4. 0.9 cc. of a 1 per cent cholesterin solution
5. 1.0 cc. of a 1 per cent cholesterin solution

Each of the cholesterinized alcoholic extracts is diluted with 5 parts of salt solution as follows: To the required amount of antigen is first added rapidly an equal amount of salt solution. Four parts of salt solution are then added and this final opalescent mixture is ready for use with serum.

The serum is inactivated for one-half hour at 56°C. and diluted 1:10 with salt solution. A dilution of 1:5 is frequently employed with somewhat sharper results. The test consists of 1 cc. of the diluted serum plus 0.5 cc. of the antigen-salt solution mixture. The results are read after 24 and 48 hours' incubation at 37°C. by means of a Kuhn-Voite Agglutinoscope.

The Sigma Reaction (11) evolved by Dreyer and Ward is a quantitative precipitation test. These workers employ an acetone insoluble antigen prepared from calf hearts ac-

cording to Bordet and Ruelens. One hundred grams of heart muscle are extracted with 125 cc. alcohol for 5 days at room temperature. The alcohol is filtered off and evaporated, 200 cc. of acetone added and extraction continued for 7 days. The acetone is filtered off and 100 cc. fresh acetone added and extraction again continued for 1 day. After filtering off the acetone, the residue is dried for several hours and dissolved in 100 cc. alcohol. This extract is cholesterinized by adding 0.25 cc. of a 1 per cent cholesterin solution in alcohol to 5 cc. of the extract. Two salt solution suspensions of this antigen are employed in the tests. The first suspension is prepared by adding 10.7 cc. salt solution to 1 cc. of extract; the second, by adding 34 cc. of salt solution to 1 cc. of extract. The salt solution is dropped from a distance of 36 cm. into the extract, at such a rate that 34 cc. will be delivered in 4 minutes and 30 seconds.

Nine varying serum dilutions are employed and the reading made with a specially devised agglutinoscope, after 7 hours' incubation in the water bath. The results indicating the strength of the reaction are expressed in standardized "sigma" units.

Dold (12) reported a turbidity reaction which, in his opinion, has two advantages over the Sachs-Georgi and Meinicke reactions, namely, the earlier reading of results and non-employment of an agglutinoscope. His antigen is similar to that employed by Sachs and Georgi and is diluted for the tests in the proportion of 1:10 with salt solution. This dilution is carried out slowly and with care so that the final mixture is cloudy and opalescent. For the test, 0.4 cc. of serum is mixed with 2 cc. of diluted extract and incubated for 2 hours at 37°C., followed by 2 hours at room temperature. At the end of this period, syphilitic sera show turbidity, while normal sera remain clear.

Hecht (13) who had already reported a precipitation reaction for syphilis in 1915 presented a new reaction, the sensitiveness of which is based on fractional dilution of antigen with salt solution. He dilutes the extract antigen 1:5 or 1:6 with salt solution and permits the mixture to stand for one-half hour to "ripen." After this period, he dilutes it further with salt solution from 1:7 to 1:14 as determined by titration with positive sera. For the test, he employs 0.1 cc. inactivated serum with 1 cc. of diluted extract. The incubation period is 8 hours.

Wang (14) has reported a precipitation test in which is employed a non-cholesterinized antigen prepared from human heart muscle. He adds 3 parts of alcohol to 1 part of ground muscle and extracts for 3 days at room temperature. For the test, 1 part of extract is diluted with 9 parts of salt solution. This dilution is carried out with great care by floating the extract on the salt solution and gently rotating at intervals. The final emulsion is of uniform turbidity and, after shaking, is ready for use. This antigen emulsion is mixed with salt solution and inactivated serum in such proportion that the amount of serum is one-twelfth of the total in 1 case and one-twenty-fourth in the other. The tubes are incubated for about 20 hours at 37°C. after which precipitates are noted.

Bruck (15) has recently gone back to precipitation studies and presented a test in which neither the incubator nor agglutinoscope were employed. His technic is as follows: Ground heart muscle is extracted with 5 parts of alcohol for 14 days. To 50 cc. of the alcoholic extract are added 25 cc. salt solution in 0.5 cc. quantities, shaking after each addition. Twenty-five cubic centimeters of salt solution are then added rapidly and mixed. The mixture is turbid and will keep well for several months. A precipitate of ten settles on the bottom of the flask after standing. The mixture is shaken before using in order to render the emulsion uni-

form. The test is carried out as follows; 0.2 cc. inactivated serum is added to 0.8 cc. Ten per cent salt solution plus 0.2 cc. of antigen-emulsion and the mixture centrifuged for 20 minutes. A precipitate is found to be suspended in both positive and negative sera. The tubes are shaken and the precipitates in negative sera go back into solution and those in positive sera are unaffected.

Meinicke (16) recently published a turbidity reaction. The antigen is the same as that employed in the "third modification." Two parts of extract are mixed with 3 parts of alcohol and to 10 cc. of this mixture is added from 1 to 1.5 cc. of a 1 per cent cholesterin solution in alcohol. The amount of cholesterin is determined by titration. To a titrated cholesterinized extract are added various amounts of a 5 per cent alcoholic solution of Balsam Tolutanum and the extract is again titrated with positive and negative sera.

The test is carried out as follows: A given amount of cholesterin-balsam extract is placed in a tube, 10 times the amount of 2 per cent salt solution is quickly added, and the two solutions thoroughly mixed. One cubic centimeter of this emulsion is mixed with 0.4 cc. of active or inactive serum and the mixture incubated for 1 hour at 37°C. The positive sera become heavily clouded and do not permit light to pass through them, while the negative sera are opalescent and permit light to pass through. The final reading is made after 2 hours' further incubation.

More recently, Meinicke and Grün (17) published a turbidity reaction employing the same balsam extract as used in Meinicke's original turbidity reaction, but cholesterin-free. This extract is mixed with 10 times the amount of 2 per cent salt solution. It is required that both solutions be at 37°C. when mixing. For the test, 1 cc. of this antigen mixture (still warm) is added to 0.4 cc. of inactivated serum. After 1 hour's incubation at 37°C., the positive sera are

cloudy and do not permit light to pass through them, while negative sera are relatively clear.

Resinous matter in connection with a precipitation test is also utilized by Dujarric de la Rivière and Gallerand (18). These authors proposed a precipitation test in which Bordet's antigen is mixed with an alcoholic solution of resin of benzoin. The readings are made after three hours' incubation at 37°C.

The Vernes reaction has aroused considerable interest in recent years. This reaction requires in its manipulation special apparatus devised by Vernes. A detailed discussion of the method is presented by Cornwall (19).

PART II

GOVERNING PRINCIPLES OF PRECIPITATION
AND THEIR APPLICATION TO
AUTHOR'S TEST

CHAPTER III

OBSERVATIONS LEADING TO DEVELOPMENT OF TEST

While working with the Meinicke and Sachs-Georgi reactions in the latter part of 1921, it soon became apparent that these tests possessed a serious practical difficulty. It was repeatedly impossible to differentiate between positive and negative results because of bacterial contamination.¹ It seemed apparent that this contamination resulted from the high dilution of the serum-antigen mixtures combined with the prolonged incubation periods employed in both tests. Knowing the inhibitory effect of serum on bacterial growth, it was thought that the degree of contamination might possibly be reduced by increasing the concentration of the serum-antigen mixtures. This could be readily accomplished by decreasing the amount of salt solution used in the tests. Since, however, salt solution is used liberally in practically all precipitation tests for syphilis, the question came up whether the element of dilution is an essential factor in the phenomenon of precipitation and whether concentration might conceivably delay or inhibit the formation of precipitates.

RECOGNITION OF IMPORTANCE OF CONCENTRATION OF INGREDIENTS

In order to compare the effects of concentration and dilution on precipitation in syphilis, it was necessary to evolve some precipitation procedure in which serum and

¹ In fairness to these precipitation tests, it should be stated that the sera employed in these tests were the routine specimens received through the mails. Age combined with lack of sterility in many cases undoubtedly explains the large number of contaminations encountered.

antigen could be used in an undiluted form. Such a procedure, it was believed, might indicate whether it was possible to obtain precipitates under conditions of high concentration, particularly without the use of salt solution.

In seeking an antigen for a method based on concentration, it appeared that neither the Sachs-Georgi nor the Meinicke antigens were especially desirable; the first, because of its high dilution—wet heart muscle being used in its preparation; and the second, because of its especial adaptation to the Meinicke Reaction, in which 2 per cent salt solution and distilled water play important rôles in the use of this antigen in the test. Two other antigens which have been in use for some years in connection with the Wassermann test were chosen—a Noguchi (20) or acetone-insoluble antigen prepared from dried beef heart and an antigen prepared according to Neumann and Gager (21) which consists of an alcoholic extract of dried beef heart from which the ether extractives have been previously removed. In order to further increase the lipid content of these antigens, 0.4 per cent cholesterin was added.

The following procedure suggested itself: To add given amounts of syphilitic and non-syphilitic serum to the undiluted antigens in such proportions as to eliminate the possibility of protein precipitation by the alcohol. Preliminary experiments soon showed that one could safely add 10 parts or even 5 parts of serum to one part of 95 per cent alcohol without throwing down the serum proteins, provided the ingredients were rapidly mixed when added to one another. Experiments further showed that when antigen, instead of alcohol, was mixed with non-syphilitic serum in the proportions of 10 and 5 parts of serum to 1 part of antigen there was also no tendency for the formation of precipitates. Having thus determined the proportions of serum and alcohol and of serum and antigen which

apparently did not give non-specific precipitation, it was possible to study specific precipitation of antigen with syphilitic serum.

Experiment. Ten sera were chosen, 5 from syphilitic patients giving positive Wassermann reactions and 5 from non-syphilitic patients giving negative Wassermann reactions. A Neumann and Gager antigen was employed. Thirty small test tubes were set up, 3 tubes for each serum. To each series of 3 tubes were added respectively 0.02 and 0.01 cc. of antigen and 0.02 cc. of 95 per cent alcohol. These amounts were pipetted to the bottom of the tubes with a 0.2 cc. pipette graduated in 0.001 cc. Each of the 3 tubes of every series now received 0.1 cc. of undiluted serum and was immediately shaken to insure thorough mixing of serum with antigen or alcohol. Prompt observation showed all the sera to be free from precipitates. Further observations were made after 1, 2, 4 and 18 hours' incubation at 37.5°C.

The results seemed at once to contradict the hypothesis that concentration may interfere with precipitation in syphilitic sera. The positive sera showed precipitation and clumping after 1 to 4 hours' incubation, while the negative sera remained practically free from precipitates even after 18 hours' incubation. The results of the experiment are illustrated in the findings with 1 negative and 3 positive sera shown in table 1.

That these findings were not accidental in character was soon established by the fact that 20 positive and an equal number of negative sera were examined with the same procedure employing both the Neumann and Gager and Noguchi antigens side by side. The results were practically the same as in the experiment described except that the Noguchi antigen gave somewhat weaker results with the positive sera and that occasionally, after incubation, there was weak non-specific precipitation in the serum-alcohol controls.

TABLE 1
Specific precipitation in syphilitic sera with concentrated antigen in absence of salt solution

	SERUM 1 (POSITIVE)			SERUM 2 (POSITIVE)			SERUM 3 (POSITIVE)			SERUM 4 (NEGATIVE)		
	0.02	0.01		0.02	0.01		0.02	0.01		0.02	0.01	
	0.1	0.1	0.02	0.1	0.1	0.02	0.1	0.1	0.02	0.1	0.1	0.02
	Results											
Incubation periods..... 1 hour 2 hours 4 hours 18 hours	++	++	+	++	++	++	++	++	+	+	+	+
	++	++	+	++	++	++	++	++	+	+	+	+
	++	++	+	++	++	++	++	++	+	+	+	+
	++	++	+	++	++	++	++	++	+	+	+	+
	++	++	+	++	++	++	++	++	+	+	+	+

Further experimentation was necessary to establish with reasonable certainty that concentration does not interfere with precipitation. It was conceivable that in the experiment just described the employment of normal salt solution in connection with the serum-antigen mixtures would either have hastened precipitation or rendered the precipitates heavier. The following experiment was thereupon carried out.

Experiment. Ten sera, 5 giving positive and 5 giving negative reactions, were employed using a Neumann and Gager antigen. The serum-antigen tests as well as the serum-alcohol controls were run exactly as described in the previous experiment. The tests with each of the 10 sera were set up in triplicate. Immediately after adding the antigen and serum quantities to all tubes, 1 set of tests received 1 cc. of salt solution in each tube, another set 0.5 cc. salt solution, and the third set received no salt solution. The results in this experiment were read after 1, 2, 4, and 18 hour's incubation at 37.5°C. The findings with the 2 positive sera shown in table 2 are typical.

It is obvious from the results of this experiment that dilution with salt solution does not enhance but actually delays and possibly inhibits precipitation in syphilitic sera. Repetition of this experiment with a Noguchi antigen gave somewhat weaker reactions with the positive sera, but the results as a whole were the same as obtained with the Neumann and Gager antigen.

The foregoing experiments led us to conclude that concentration tends to hasten and intensify precipitation in syphilitic serum-antigen mixtures, while dilution with salt solution tends to delay and weaken precipitation. These experiments further indicated that a precipitation test for syphilis which is to possess practical value must be based on concentration.

TABLE 2
Delaying effect of salt solution on specific precipitation in syphilitic sera

	SERUM 1 (POSITIVE)							SERUM 2 (POSITIVE)						
	0.02	0.01		0.02	0.01		0.02	0.01		0.02	0.01		0.02	0.01
Antigen, cc.....														
Alcohol (control), cc.....			0.02			0.02						0.02		0.02
Serum, cc.....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Salt solution, cc.....	0.0	0.0	0.0	0.5	1.0	1.0	0.0	0.0	0.0	0.5	0.5	1.0	1.0	1.0
Results														
Incubation periods..... $\left\{ \begin{array}{l} 1 \text{ hour} \\ 2 \text{ hours} \\ 4 \text{ hours} \\ 18 \text{ hours} \end{array} \right.$	++++	++++	-	+	+	-	++++	+	+	+	+	+	-	-
	++++	++++	-	+++	+	-	++++	+	+	+	+	+	-	-
	++++	++++	-	++++	+	-	++++	+	+	+	+	+	+	-
	++++	++++	-	++++	++++	++	++++	++++	++++	++++	++++	++++	++++	+

EARLY RECOGNIZED REQUIREMENTS FOR A PRACTICAL PRECIPITATION TEST

The question that presented itself at this time was whether it was possible to evolve a practical precipitation test for syphilis by using undiluted antigen with serum in a manner described in the foregoing experiments. Over 100 examinations with sera from different stages of syphilis employing both the Neumann and Gager and Noguchi antigens convinced us that, at best, a test of this kind would be presumptive in character. Many of the weaker sera did not show precipitation with this method even after prolonged incubation and occasionally a serum-alcohol control showed the presence of a precipitate. It seemed possible that better results would be obtained by diluting the antigen with salt solution—using an amount, however, that would be sufficiently small not to delay precipitation. What appeared to be desirable, therefore, in the development of a precipitation test was the use of (a) a concentrated antigen, (b) undiluted serum and (c) a minimum amount of salt solution for antigen dilution.

Antigen. In attempting to choose some antigen of sufficient concentration, preliminary experiments soon showed that an antigen prepared from wet heart muscle would not be likely to form the basis of a practical test. When employing such antigens with syphilitic sera, prolonged incubation was necessary, undoubtedly due to the dilution caused by the high water content of the wet muscle. It was believed that the comparatively higher concentration possible in an antigen prepared from dried beef heart might be such as to reduce the period of incubation.

The use of an alcoholic extract antigen prepared from dry powdered beef heart seemed promising. Such an antigen was prepared by adding 5 parts of 95 per cent alcohol to 1 part of powdered heart muscle and extracting for 5 days at 37.5°C. After filtration, the extract was

permitted to stand for a day at room temperature in order to throw down the precipitate which usually forms when an antigen extracted in the incubator is placed at a lower temperature. To this clear extract was added 0.4 per cent cholesterolin before using in the tests.

Serum. The sera to be tested were employed in undiluted form and, as in the Wassermann test, after heating for 30 minutes at 56°C.

Antigen dilution. The dilution of the antigen with salt solution was carried out with the well known fact in mind that salt solution added slowly to antigen will tend to produce mixtures containing a precipitate, whereas salt solution added rapidly to antigen will, within certain limits, produce mixtures which are opalescent. It was desired to have an antigen-saline mixture which was opalescent, for it did not seem at that time that a mixture containing a precipitate could possibly be used in a precipitation test. It was also desired that the antigen-saline mixture should contain a minimum amount of salt solution. By diluting equal amounts of antigen with varying amounts of saline, it was soon found that 7.5 parts represented the minimum amount of salt solution which would produce an opalescent mixture.

It had been previously observed, however, that after rapidly adding salt solution to the Noguchi or the Neumann and Gager antigens one could obtain opalescent mixtures with only about 2.5 to 3 parts of salt solution to 1 part of antigen. Believing that the less salt solution employed for dilution purposes the more rapid the formation of precipitates, it seemed likely that these two purified antigens diluted with less salt solution might give better results than the crude alcoholic extract with comparatively larger amounts of salt solution.

Experiment. The object was to find the relative sensitiveness with syphilitic sera of a crude alcoholic extract of

TABLE 3

Comparative sensitiveness to precipitation with syphilitic serum of crude alcoholic and refined extract antigens

ANTIGEN—PREPARED FROM POWDERED BEEF HEART									
		Crude alcoholic extract		Acetone insoluble extract		Alcoholic extract after preliminary ether extraction			
		1 + 7.5		1 + 2.5		1 + 2.5			
Dilution of antigen with salt solution resulting in opalescent mixtures.....		0.05	0.025	0.05	0.025	0.05	0.025	0.05	0.025
		0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
		3:1	6:1	3:1	6:1	3:1	6:1	3:1	6:1
Results									
Incubation periods									
Serum 1.....	None	+	—	++	+	++	—	++	+
	1 hour	++	+	++	+	++	++	++	+
	3 hours	+++	++	+++	+	+++	++	+++	+
	18 hours	+++	++	+++	+	+++	++	+++	+
Serum 2.....	None	—	—	—	—	—	—	—	—
	1 hour	—	—	—	—	—	—	—	—
	3 hours	+	±	+++	+	+++	++	+++	+
	18 hours	+++	++	+++	+	+++	++	+++	+
Serum 3.....	None	—	—	—	—	—	—	—	—
	1 hour	—	—	—	—	—	—	±	—
	3 hours	—	—	—	—	—	—	+++	++
	18 hours	+++	++	+++	+	+++	++	+++	++

powdered beef heart diluted with salt solution in the proportion of 1 + 7.5 parts and two purified extract antigens (Neumann and Gager and Noguchi), each diluted with salt solution in the proportion of 1 + 2.5 parts. The amount of salt solution in each case represented the least which would produce an opalescent solution with the antigen. Ten sera were tested, 8 positives and 2 negatives. The antigen-saline mixtures were used in 0.05 and 0.025 cc. quantities. The sera were employed in 0.15 cc. amounts. The tests were run in triplicate, employing the 3 antigen mixtures with each serum. The findings with 3 sera shown in table 3 illustrate the results obtained in this experiment.

The Neumann and Gager antigen appeared most sensitive, the Noguchi somewhat less so, and the crude alcoholic extract least sensitive. Repetition of these experiments with 20 positive and 20 negative sera gave similar results. Still further experiments indicated the superiority of the Neumann and Gager antigen both from the standpoint of sensitiveness and apparent specificity and led us to choose this antigen for the basis of a practical precipitation test.

AUTHOR'S EARLY PRECIPITATION PROCEDURE

Having decided upon an antigen, it was desired to determine the conditions under which extracts would give constant results. It was soon observed that greater uniformity of the alcoholic extract was obtained by using definite amounts of beef heart and alcohol, rather than average amounts indicated by Neumann and Gager. A proportion of 1 gram of powdered heart muscle to 5 cc. of 95 per cent alcohol was thereupon adopted—after determining that the antigens prepared by using this proportion gave good results.

When testing this antigen with sera it was diluted with the minimum amount of salt solution which resulted in an

opalescent mixture, most antigens requiring 3 parts of salt solution. In some cases, 2.5 parts of salt solution produced mixtures which were opalescent and could, therefore, be used in the tests. In practice the antigen and saline were usually mixed in comparatively large amounts—such as 25 cc. antigen and 75 cc. saline—and this diluted antigen used from day to day as it was needed. A study of the quantitative relation of serum and antigen dilution indicated that with strongly positive sera good results could be obtained by using equal amounts of serum and antigen dilution. Weaker sera, however, gave stronger reactions with less antigen dilution and, in order to fulfill the demand of the larger number of sera, a 3:1 proportion of serum to antigen-dilution was adopted.

In brief, the following was the author's preliminary precipitation procedure. The antigen was prepared from powdered beef heart by first extracting three or four times with ether at icebox temperature until the supernatant ether was practically free from coloring matter and subsequently extracting with 95 per cent alcohol in the proportion of 1:6, for 10 days in the icebox. To each cubic centimeter of alcoholic extract were added 4 mgm. of cholesterin. The antigen was diluted with 3 or 2.5 parts of salt solution depending on the proportion representing the minimum amount producing an opalescent mixture. In most cases, 3 parts of salt solution mixed with 1 part of antigen gave desired results. Each test consisted of 0.1 cc. antigen-dilution and 0.3 cc. inactivated serum. After adding the serum to the antigen dilution and placing the tubes in the water bath, those which gave heavy precipitates at the end of 1 hour were considered "strongly positive;" those which required 3 hours for the formation of a heavy precipitate were "positive;" and those which showed weak precipitates at the end of this period were "weakly positive."

After 1100 comparative tests with the Wassermann, we considered this simple procedure of sufficient value as a check on the Wassermann method to publish a preliminary report.

Soon it was observed that a non-cholesterinized antigen could also be used in a test of this kind, provided the same principle employed in connection with the cholesterinized antigen was abided by, namely, to employ in the dilution of the antigen a minimum amount of salt solution which would produce an opalescent mixture. This cholesterol-free extract could usually be diluted with 2 in-

TABLE 4
Early procedure of author's precipitation test

	TUBE 1	TUBE 2	COMPLETION OF TEST
	cc.	cc.	
Cholesterinized antigen + saline (1 + 3).....	0.05		The racks are shaken for three minutes. Strongly positive sera show immediate reactions. Final reading is made after overnight incubation
Alcoholic antigen + saline (1 + 2).....		0.05	
Serum (undiluted).....	0.3	0.3	

stead of 3 parts of salt solution and gave good results when 0.1 cc. was used with 0.3 cc. of serum and the mixture incubated over night at 37°C.

It was further observed that antigens, although prepared similarly, did not always possess the same degree of sensitiveness, particularly with weaker sera, and that overnight incubation of the tests was necessary for more sensitive precipitation results. The procedure subsequently adopted included the employment of both a cholesterinized and non-cholesterinized antigen and overnight incubation. There was practically no tendency for contamination, presumably because of the high degree of concentration. Furthermore, the strongly positive reactions, instead of show-

ing precipitates in the usual sense of the term, showed one or more clumps which rendered the reading of the results comparatively easy.

ELIMINATION OF INCUBATION

Although the early precipitation method just described proved a valuable check on the Wassermann in this and in a number of other laboratories, it failed to meet the demands of a fully acceptable test in that a prolonged incubation period was necessary to bring about precipitation in weaker sera and there were occasional negative reactions in sera from unquestionable cases of syphilis. Experience gained thus far with the phenomenon of precipitation led us to believe that, if we could produce an antigen-saline mixture of sufficient concentration as well as sufficient instability, we could probably eliminate the element of incubation as well as increase the sensitiveness of the test. These observations will be discussed more fully under instability of antigen dilution (Chapter V). Briefly, it was observed that certain proportions of antigen and salt solution, such as equal parts, produced precipitates capable of readily dissolving in serum. The use of an amount of salt solution for dilution of the antigen approximately equal to that of the antigen reduced the salt solution to one-third of that employed in the earlier procedure. Furthermore, an antigen-salt solution mixture containing a precipitate capable of dissolving in serum, suggested an unusually high degree of instability.

When this antigen-saline mixture was added to syphilitic and non-syphilitic sera an unusual phenomenon was observed. The antigen precipitates immediately went into solution. In syphilitic sera a new and apparently specific precipitate became visible within some minutes. Further studies fully corroborated this observation and it seemed clear that incubation was not a necessary element in the precipitation phenomenon in syphilis.

THE AUTHOR'S PRESENT PRECIPITATION METHOD

Having determined conditions under which incubation was unnecessary to bring about precipitation in serum-antigen mixtures, we had the beginning of a practical precipitation method. The perfection of such a method, however, required a quantitative study of various problems.

First, it was necessary to experimentally establish the conditions governing the preparation of an antigen-saline dilution of required sensitiveness, as well as those governing the uniformity of such a dilution when prepared by different workers. Second, it was important to determine the proper proportions of serum and antigen dilution which would give optimum precipitation reactions. We had observed that our antigen dilution, while highly sensitive when mixed with definite proportions of syphilitic serum, was capable of giving negative results when these proportions were changed even in a comparatively small degree.

Then again, the element of agitation or shaking of the serum-antigen mixtures required study. From our earliest attempt to develop a precipitation test based on concentration, we observed that shaking of the serum-antigen mixtures hastened the formation of precipitates. This might have been expected in view of the fact that we were dealing with colloidal mixtures in which the motion of the particles would likely be slow. With our new and highly sensitive antigen dilution it became of importance to determine to what extent shaking hastened precipitation in positive sera and whether it was capable of producing precipitates in negative sera. These and several related problems will be only touched upon here since they are fully discussed in forthcoming chapters.

Dilution of antigen with salt solution. Our observations indicated that several antigen-saline proportions produced precipitates readily soluble in salt solution and gave sensitive precipitation reactions with syphilitic serum. Thus,

when 1 cc. quantities of antigen were diluted with 1 and 1.5 cc. amounts of salt solution, respectively, both dilutions contained precipitates and gave sensitive reactions. The degree of sensitiveness, however, could only be determined by special experiments. These soon indicated that the more sensitive antigen dilution was the one containing the smaller amount of salt solution. After determining that such an antigen dilution gave apparently specific results, it was chosen for use in the tests.

Quantitative relation between serum and antigen-saline dilution. Preliminary experiments suggested that sera from different stages of syphilis required different amounts

TABLE 5
Outline of routine test

TUBE NUMBER	ANTIGEN- SALINE DILUTION	SERUM (UNDI- LUTED)	COMPLETION OF TEST
	cc.	cc.	
1	0.05	0.15	All tubes are shaken for two minutes, after which 0.5 cc. saline is added to each tube and the final reading is made
2	0.025	0.15	
3	0.0125	0.15	

of antigen-dilution for complete precipitation reactions. In order to conform with this requirement, the employment of several proportions of serum and antigen dilution was necessary. Further experiments indicated that the three proportions of serum to antigen dilution, 3:1, 6:1 and 12:1, would be likely to meet the requirements of a practical test. These proportions were thereupon adopted in the routine test.

It should be indicated in this connection that by the term "routine test" there will also be assumed the use of an antigen containing 0.6 rather than 0.4 per cent cholesterol. A discussion of the rôle of cholesterol will be presented in connection with studies on uniformity in antigen preparation (Chapter VII).

Finally, experiments on agitation of the serum-antigen mixtures indicated that a two minute shaking period would meet the requirements of the larger number of sera and was thereupon adopted as a step in the routine test.

Table 5 gives an outline of this test. Although a detailed discussion of the important steps will be presented under separate headings, the outline is given at this time because of the frequent references to the method in the text.

CHAPTER IV

RÔLE OF CONCENTRATION

Although the importance of concentration has been discussed in connection with its bearing on the development of the author's test, there are several phases of the problem worthy of further consideration. Having observed that an excessive amount of salt solution delays precipitation, it seemed of interest to determine the factors responsible for the delaying effect. It was also of interest to study the relation between concentration and agglutination of the precipitated particles. These and several associated problems will now be considered.

DELAYING EFFECT OF SALT SOLUTION ON PRECIPITATION

Preliminary experiments established that if varying amounts of salt solution were added either to the serum alone or to the antigen-saline dilution alone and the test subsequently completed, there was delay in the formation of precipitates. This delay was found to be directly proportional to the amount of salt solution added. It was thereupon desired to find to what extent the addition of salt solution was capable of delaying precipitation if added after mixing serum with antigen—just before the appearance of a precipitate.

Experiment. Ten positive and 5 negative sera were used in this experiment. Five regular tests were carried out with each serum. Test 1 was carried out in the usual manner by adding to three tubes 0.05, 0.025 and 0.0125 cc. antigen-saline dilution, respectively, followed by 0.15 cc. serum. No salt solution was added to the tubes of this test. Tests 2, 3, 4 and 5 were carried out in the same

manner as test 1 except that 0.15, 0.45, 1.05 and 1.65 cc. salt solution, respectively, were added to them. The racks were shaken two minutes to permit thorough mixing and

TABLE 6

Effect of dilution followed by various periods of incubation on precipitation of syphilitic serum and antigen

PERIOD OF INCUBATION AFTER MIXING SERUM AND ANTIGEN DILUTION	DILUTION WITH 0.85 PER CENT SALT SOLUTION	SERUM : ANTIGEN DILUTION		
		3:1	6:1	12:1
		Results		
hours	cc.			
0	0.0	++++	++++	++++
	0.15	+	++	++
	0.45	—	+	+
	1.05	—	—	—
	1.65	—	—	—
2	0.0	++++	++++	++++
	0.15	++	++++	++++
	0.45	±	++	++
	1.05	—	—	—
	1.65	—	—	—
4	0.0	++++	++++	++++
	0.15	+++	++++	++++
	0.45	++	++++	++++
	1.05	+	++	++
	1.65	—	+	+
24	0.0	++++	++++	++++
	0.15	++++	++++	++++
	0.45	+++	++++	++++
	1.05	++	+++	+++
	1.65	++	++	++

the results were read immediately and after 2, 4 and 24 hours' incubation. Table 6 gives the results obtained with 1 serum, which, however, is representative of the rest of the positive sera studied.

Table 6 points definitely to the fact that, even after mixing serum with antigen dilution, salt solution is capable of delaying the formation of precipitates in the author's test. It indicates another interesting fact in connection with precipitation in syphilitic sera, namely, that sufficient incubation of strongly positive sera may, in a large measure, counterbalance the delaying effect of dilution. In the case of weakly reacting sera, however, a period as long as 48 hours' incubation is frequently insufficient to overcome the delaying effect of 1.65 cc. of salt solution.

The question now arose as to the factor responsible for the delaying effect of salt solution on precipitation. It was not likely that sodium chloride, an electrolyte known to play an important rôle in the formation of precipitates, would be the delaying factor. Rather, it was believed that the dilution element was the underlying cause of the delay. In order to test the validity of this view, the following experiment was carried out.

Experiment. Five different positive sera were divided into three parts. Five regular tests were subsequently run with each part. The first of these tests in each case consisted of regular serum-antigen proportions which were examined for precipitates without further dilution. The remaining 4 tests contained the same serum-antigen proportions and were examined for precipitates after adding 0.15, 0.45, 1.05 and 1.65 cc. distilled water, respectively, to one series, the same amount of 2 per cent salt solution to another; and the same amounts of 0.85 per cent salt solution to the third series. The results are indicated in table 7.

The findings, in our opinion, are of especial interest because they indicate not only that the delay in precipitation is due to the pure dilution element caused by the water but that the enhancing effect of sodium chloride on precipi-

tation is neutralized by the dilution factor when comparatively large amounts of salt solution are used.

EFFECT OF CONCENTRATION UPON AGGLUTINATION OF PRECIPITATE

In our earlier precipitation experiments, it was observed that in concentrated mixtures we obtained not only precipitates but ready clumping of these precipitates. This pointed to the possibility that in the precipitation phenomenon in syphilis we were dealing with two phases: preliminary precipitation and the agglutination of the precipitate. It also indicated that concentration affected not only the formation of precipitates but their agglutination as well. The following experiment was carried out with a view of observing to what degree the clumping of precipitates was influenced by concentration.

Experiment. Six positive sera were employed; 0.15 cc. of each of the sera were measured into six series of 4 tubes each, after they had received 0.025 cc. antigen dilution. All tubes were shaken 2 minutes in the usual manner and observed for precipitates—which were evident in every case; 0.5, 1 and 1.5 cc. salt solution respectively were then added to 3 of the tubes of each series, leaving 1 tube for a control. The results were read after 1, 4 and 24 hours at 37°C. It was found that the tubes which received no salt solution showed complete clumping of the precipitates after 1 hour incubation, while the tubes containing salt solution showed delay in clumping directly proportional to the amounts used. Three of the sera showed no clumping even after 24 hours' incubation where the 1 and 1.5 cc. salt solution were used. Only the remaining 3 strongly positive sera gave evidence of clumping after 24 hours' incubation with the larger amounts of salt solution.

It seemed apparent that the clumping of precipitates was markedly delayed and, in some cases, inhibited by salt

solution. It was further observed that salt solution added to clumps already formed immediately broke them up into smaller particles and, when added in excess, actually rendered the precipitate so fine as to make it hardly visible.

It would appear that the formation of precipitates in syphilitic serum and antigen is closely allied with the clumping of the precipitates—that the precipitate when formed is probably fine and invisible; but the affinity of the particles for one another is so marked that agglutination immediately takes place and thus renders the particles visible. It is interesting to note that sera from different stages of syphilis, particularly from cases under treatment, show different degrees of this agglutinating power. Thus, a serum from an untreated case of secondary syphilis may possess such marked clumping ability that the precipitate will form into a single clump within an hour after mixing serum with antigen. The serum from the same case after treatment may still show a heavy precipitate, but some of its agglutinating power will be lost, so that it may require prolonged incubation for complete clumping; after still further treatment, there will be no further clumping even after prolonged incubation. It is not unlikely, that what we term a weak precipitate is not due to incomplete precipitation of the serum-antigen mixture but rather to insufficient clumping. It is clear that the agglutination phase plays an important rôle in what we term the precipitation phenomenon in syphilitic sera and that concentration markedly enhances this phase.

CONCENTRATION VERSUS DILUTION IN THREE DIFFERENT PRECIPITATION TESTS

Finally, in summing up the rôle of concentration in the phenomenon of precipitation in syphilitic sera, it seems worth while to indicate diagrammatically the effect of dilution on two well known precipitation tests for syphilis compared with the effect of concentration on the author's test.

TABLE 8
Dilution versus concentration in three precipitation reactions for syphilis

	MEINICKE	SACHS-GEORGI	KAHN
Antigen preparation	Base.....	Beef heart—not dried	Beef heart—dried
	Extraction.....	Alcoholic—direct. Subsequently diluted 1 to 3 times with alcohol according to special titration	Alcoholic after several preliminary ether extractions
	Cholesterin....	Approximately 0.15 per cent	0.6 per cent
Dilution of antigen for tests.....	1 part antigen + 0.5 part distilled water + 7 parts 2 per cent salt solution	1 part antigen + 5 parts 0.85 per cent salt solution	1 part antigen + 1 part 0.85 per cent salt solution (based on titration)
Diluted antigen used in tests, cc.....	0.8	0.5	0.05, 0.025, 0.0125
Serum used in tests, cc.....	0.2	0.2 (1 cc. 1:5 dilution)	0.15, 0.15, 0.15
Incubation period required.....	24 (and 48) hours	24 (and 48) hours	Readings made immediately

CHAPTER V

INSTABILITY OF ANTIGEN DILUTION

Next in importance to concentration in the precipitation phenomenon in syphilis, is instability of the antigen-saline mixture. It is to be expected that the union of the reacting substances of syphilitic serum and antigen will take place more readily when the antigen-saline mixture is in a state of instability rather than stability. We observed again and again in our earlier precipitation procedure, when we employed an opalescent antigen dilution consisting of one part of antigen and three parts of salt solution, that the antigen dilution which spontaneously precipitated on standing—indicating instability with reference to precipitation—produced highly sensitive reactions when mixed with syphilitic sera. On the other hand, an antigen-saline dilution which remained opalescent invariably produced reactions of lesser sensitiveness when mixed with syphilitic sera.

AN ANTIGEN-SALINE PRECIPITATE SOLUBLE IN SERUM

While studying the nature of the precipitates formed on mixing various amounts of antigen and salt solution, several observations were made which led to the evolvment of a highly unstable antigen dilution and which, in turn, we believe led to the elimination of the incubation period.

Experiment. One cubic centimeter quantities of cholesterinized antigen used in the test were added to a series of four tubes; 3, 2, 1 and 0.5 cc. salt solution, respectively, were added to these tubes and the antigen and saline mixed uniformly in each case. It was observed that the tube containing 1 cc. antigen and 3 cc. saline showed no precipitate and the remaining tubes showed precipitates. These pre-

cipitates, however, after removal by centrifugation, showed marked differences in their ability to redissolve on addition of salt solution. Thus, the precipitates formed on mixing 1 cc. antigen with 2 or 1 cc. salt solution readily dissolved, whereas the precipitate formed on mixing 1 cc. antigen with 0.5 cc. saline did not dissolve in salt solution. Table 9 gives an outline of this observation.

TABLE 9

Solubility of precipitates resulting from the dilution of antigen with varying amounts of salt solution

	TUBE 1	TUBE 2	TUBE 3	TUBE 4
Antigen + salt solution, cc...	1 + 3	1 + 2	1 + 1	1 + 0.5
Appearance of resulting mixture.....	Opal- escent	Precipi- tate present	Precipi- tate present	Precipi- tate present
Precipitates separated by centrifugation and tested for solubility in salt solution				
Results.....		Soluble	Soluble	Insoluble

It was further observed that the solutions formed after dissolving the precipitates of tubes 2 and 3 (table 9) in salt solution brought about specific precipitation with syphilitic sera after incubation. The dissolved precipitate of tube 3, containing the more concentrated mixture, gave the more sensitive precipitation reactions. When 0.05 cc. of this solution was employed with 0.3 cc. serum and the mixture incubated, apparently specific precipitation results were obtained. The use of this dissolved precipitate suggested itself as a check on the 1 + 3 antigen dilution employed in our earlier method and was actually employed in that capacity for some months in this laboratory. We thus had two cholesterinized antigen procedures and one cholesterin-free antigen procedure—all giving apparently

good results, but all requiring overnight incubation for the weakly reacting sera.

It finally occurred to us that, if a precipitate produced by mixing equal amounts of antigen and salt solution was capable of readily dissolving in salt solution, the same precipitate would undoubtedly also be capable of going into solution in serum. If so, we might conceivably have an antigen-saline mixture of unusual sensitiveness; for such an antigen mixture would conform with the two conditions which our experience had already indicated were essential for sensitive precipitation reactions in syphilis, namely, instability with reference to precipitation and a high degree of concentration. An antigen-saline precipitate which readily dissolved on further addition of saline appeared to us unstable in an unusually high degree. The use, furthermore, of approximately 1 part instead of 3 parts of salt solution in the dilution of the antigen reduced the salt solution to a practically insignificant amount.

That this antigen-saline dilution possessed a high degree of sensitiveness was soon established by actual experiments. Upon adding sera to varying proportions of the antigen dilution, the immediate effect was the solution of the antigen-saline precipitate. Within a minute or two, however, the positive sera showed new precipitates while the negative sera remained clear.

MINIMUM AMOUNT OF SALINE IN ANTIGEN DILUTION

Since antigen-saline proportions of 1 + 1, 1 + 1.5 and 1 + 2 gave precipitates which readily dissolved in serum, the question came up, what should be the criterion for antigen dilution for use in the tests? The following experiment throws light on this problem.

Experiment. One cubic centimeter amounts of antigen were diluted with 0.5, 1, 1.5, 2 and 3 cc. of salt solution, respectively. All dilutions except the last, containing 1

cc. antigen and 3 cc. saline, showed precipitates. The first (1 cc. antigen + 0.5 cc. saline) gave a precipitate insoluble in saline and therefore could not be used. The precipitates in the remaining three mixtures were readily

TABLE 10

Comparative sensitiveness to precipitation of antigen diluted with varying amounts of salt solution

		ANTIGEN-SALINE DILUTION			
		1	2	3	4
Antigen + salt solution.....		1 + 1	1 + 1.5	1 + 2	1 + 3
Antigen dilution, cc.....		0.06	0.075	0.09	0.12
Serum (undiluted), cc.....		0.15	0.15	0.15	0.15
	Incubation period	Results			
Serum 1 (positive)	None	++++	++++	++++	++++
	1 hour	++++	++++	++++	++++
	3 hours	++++	++++	++++	++++
	18 hours	++++	++++	++++	++++
Serum 2 (weakly positive)	None	++++	+	—	—
	1 hour	++++	++	—	—
	3 hours	++++	+++	+++	++
	18 hours	++++	++++	++++	++++
Serum 3 (weakly positive)	None	—	—	—	—
	1 hour	+++	—	—	—
	3 hours	++++	+	—	—
	18 hours	++++	+++	++	+
Serum 4 (negative)	None	—	—	—	—
	1 hour	—	—	—	—
	3 hours	—	—	—	—
	18 hours	—	—	—	—

soluble. All dilutions except the first were tested with 10 positive sera from different stages of syphilis. Five negative sera were used as controls. The quantities of antigen-dilution employed were 0.06, 0.075, 0.09 and 0.12 cc., re-

spectively, all these quantities containing the same amount (0.03 cc.) of undiluted antigen; 0.15 cc. inactivated serum was used in each case.

The results of this experiment are illustrated in the findings with 4 sera shown in table 10 and indicate that the antigen-saline dilutions in which equal proportions of antigen and saline were used were more sensitive than those in which the proportional amount of saline was increased.

This and similar experiments led us to recognize the following principle in connection with dilution of antigen for this precipitation test: The smallest amount of salt solution should be employed which is capable of producing a precipitate soluble on further addition of salt solution. An amount of salt solution equal to the amount of antigen usually represents the smallest amount of salt solution which meets this condition. Occasionally, however, 0.9, 1.1 or 1.2 parts of salt solution to 1 part of antigen represent the required proportions.

UNSTABLE ANTIGEN DILUTION ESSENTIAL FOR SENSITIVENESS

We have emphasized thus far two properties of the antigen dilution which apparently are responsible for its high degree of sensitiveness with sera—concentration and instability. With regard to instability we have particularly emphasized the presence of a precipitate in the antigen dilution capable of readily dissolving in serum. It has just been shown, however, that an antigen dilution of high sensitiveness must contain a minimum amount of salt solution; in other words, the antigen dilution must be of high concentration. It thus seemed possible that concentration rather than instability of the antigen dilution might be directly responsible for its high sensitiveness.

The following experiment was carried out to determine whether the presence of an unstable precipitate in the antigen dilution was directly related to immediate precip-

itation reactions with syphilitic sera. The plan of this experiment was to add a minimum amount of saline to antigen dilution—enough to dissolve the precipitate—and subsequently test this antigen dilution with a number of syphilitic sera. Further, to add a similar amount of saline to the same sera and test them with standard antigen dilution. Since the quantity of salt solution necessary to dissolve the antigen-dilution precipitate was small, the dilution effect on the serum-antigen mixture would be negligible. It was believed therefore that, if the presence of an unstable precipitate in the antigen dilution was essential for sensitiveness, the series in which the saline was added to the antigen dilution, causing solution of the antigen precipitate, would give considerably less sensitive reactions than the series in which the same amount of saline was added to the sera.

Experiment. Antigen dilution was prepared by mixing 1 cc. antigen with 1 cc. saline and then added in 0.025 cc. amounts to three series of 25 tubes. To each tube of one series was subsequently added 0.025 cc. salt solution—an amount capable of dissolving the precipitates—followed by 0.15 cc. of each of 25 different sera, in sequence. The tests were shaken 2 minutes and results read after the addition of 0.5 cc. salt solution to each tube.

In the case of the second series, instead of adding 0.025 cc. saline to the antigen dilutions, the same amount of saline was added to the 0.15 cc. serum quantities. Actually, to 0.3 cc. of each of the sera was added 0.05 cc. saline; 0.15 cc. quantities of these diluted sera were then added in sequence to the 25 tubes each containing 0.025 cc. standard antigen dilution.

The third or control series was carried out by adding 0.15 cc. amounts of each of the sera to 0.025 standard antigen dilution.

The total concentration of the two sets of tests which received the additional saline was identical, the difference being that in one set saline was added to the antigen dilution, and in the other set the same amount of saline was added to the serum, before completing the tests.

The results are indicated in table 11. It is evident from this table that in the series where the salt solution was added

TABLE 11

Comparative effect of adding a minute amount of salt solution to antigen dilution and adding the same amount to serum

NUMBER OF SERA	METHOD OF DILUTION WITH SALT SOLUTION		
	0.025 cc. saline added to 0.025 cc. antigen dilution before addition of 0.15 cc. serum	0.025 cc. saline added to 0.15 cc. serum before adding to 0.025 cc. antigen dilution	Control: 0.025 cc. antigen dilution + 0.15 cc. serum
	Results		
7	++++	++++	++++
1	+++	++++	++++
2	++	++++	++++
1	±	++++	++++
1	++	+++	+++
1	+	++	+++
2	±	++	++
1	±	+	++
1	±	+	+
3	—	+	+
1	—	±	+
3	—	±	±
1	—	—	—

to the serum, the results were practically the same as the control tests where no salt solution was added; while in the series in which the saline was added to the antigen dilution—bringing about solution of the antigen precipitate—many sera were considerably weaker than in the regular tests. This indicates that the physical character of the antigen dilution, particularly the presence of the extremely unstable precipitate, is directly related to sensitiveness of the antigen dilution.

IMPORTANCE OF UNIFORMITY IN DILUTION OF ANTIGEN

In our studies on antigen dilution, it soon became clear that it was necessary to have a standardized technic for diluting antigen with salt solution to assure uniformity in the hands of different workers. It is well known that the manner in which salt solution is added to antigen markedly affects the final product (Sachs and Rondoni). While it is true that approximately equal amounts of antigen and salt solution result in a mixture containing a precipitate which dissolves on further addition of salt solution or serum, it is also true that the antigen must be diluted with salt solution in a definite manner to bring about this condition.

One of the major requirements in diluting antigen with salt solution is that the process be carried out rapidly in order to avoid a stable or insoluble antigen-dilution precipitate. Since the degree of rapidity in diluting the antigen was likely to vary in the hands of different workers, we resorted to test tubes of a special size in order to overcome this variable element. A tube was chosen of 1.5 cm. diameter and only about 5.5 cm. in length. This tube is used for antigen dilution as follows: One cubic centimeter of antigen is measured into 1 of these tubes and approximately 1 cc. salt solution, depending on titre of the antigen, into another tube. The salt solution is poured into the antigen and, without waiting to drain the salt solution tube, the mixture is immediately poured back and forth five or six times. This antigen dilution, after a short interval, is ready for use with serum.

Experience has shown that different workers will mix antigen with salt solution correctly the first time when employing these short, wide-mouthed tubes, whereas, if longer tubes and particularly those with smaller diameters are employed, workers encounter considerable difficulty in preparing correct antigen dilutions.

These antigen-dilution tubes, furthermore, are of sufficient size to permit the dilution of 2 or 3 cc. of antigen at one time. As will be shown later, it is not well to dilute larger amounts than 3 cc. of antigen at one time because of the instability of the final product.

The dilution of lesser amounts of antigen than 1 cc. is not recommended. The reason for this is that, in attempting to pour less than 1 cc. of salt solution into the required amount of antigen, the amount of salt solution reaching the antigen in the initial pouring may be so small in relation to the amount of antigen that the resulting precipitate may be stable and insoluble in serum rather than unstable and soluble as required.

To illustrate: It will be recalled that, while equal amounts of antigen and salt solution result in an unstable precipitate, twice the amount of antigen to salt solution results in a stable or insoluble precipitate. Suppose one attempts to dilute 0.5 cc. antigen with 0.5 cc. salt solution. In the initial pouring of the salt solution into the antigen tube, only 0.3 cc. may reach the antigen, the remaining 0.2 cc. adhering to the wall of the tube. We would thus have 0.5 cc. antigen mixed with 0.3 cc. salt solution for a minimal period—sufficient perhaps to produce a mixture containing a stable precipitate. Although the mixture is immediately poured back into the original salt solution tube, the precipitate formed may be insoluble when added to serum and the antigen dilution, therefore, unfit for use in the tests.

In our experience, 0.9, 0.8 or 0.7 cc. of antigen may be diluted with salt solution with safety but the employment of less than 0.7 cc. is to be avoided. One cubic centimeter of antigen has been adopted as a standard with reference to dilution with salt solution. After dilution, the quantity will be sufficient for about 15 tests with the necessary controls.

USE OF SALINE IN STANDARDIZING ANTIGEN DILUTION

The method for standardizing or titrating antigen for this test will be fully considered in another chapter. We only wish to indicate here that we do not resort to strongly positive sera in determining the titre of a given antigen for this test. We believe that the wide use of pooled positive sera in the standardization of antigen for the Wassermann test is erroneous in principle. Sera from different stages of syphilis vary markedly in the number of reacting substances and the titration of an antigen with pooled sera or a given strongly positive serum can furnish no criterion as to the amount of antigen which might approach the optimum with the various sera employed in the tests.

The titration of a given antigen for our precipitation test is carried out with salt solution and the criterion in determining the titre is to find the smallest amount of salt solution to add to a given amount of antigen which will result in a precipitate readily soluble on further addition of salt solution. The antigen-saline dilution containing a precipitate of this character will be found to give optimum results when tested with syphilitic sera.

EFFECT OF AGE ON SENSITIVENESS OF ANTIGEN DILUTION

There is still another phase to be considered in connection with antigen-saline dilution. We have emphasized the fact that the antigen-saline dilution is extremely unstable, containing a precipitate readily soluble in salt solution or serum. Observations indicated that this precipitate does not remain soluble indefinitely. After diluting an antigen with the required amount of salt solution and permitting it to stand for about an hour or more at room temperature, cholesterol crystals appear in the antigen dilution and interfere with the recognition of specific precipitates. In order to overcome this factor and permit a

sufficient margin of safety, an antigen is so standardized as to be employed in the tests within about 30 minutes after dilution with salt solution.

It was further observed that immediately after diluting an antigen with salt solution the antigen dilution was slightly less sensitive to precipitation with syphilitic sera than a short interval after dilution. The following experiment throws light on this observation.

TABLE 12

Effect of age of antigen dilution on sensitiveness of precipitation reactions

Antigen dilution, cc.....	0.025	0.025	0.025	0.025	0.025
Serum, cc.....	0.15	0.15	0.15	0.15	0.15
Interval between dilution of antigen and use with sera, minutes.....	0	5	10	20	30
Serum number	Results				
1	++++	++++	++++	++++	++++
2	+	+	++++	++++	++++
3	+	++	++	+++	+++
4	++	+++	++++	++++	++++
5	++++	++++	++++	++++	++++
6	±	++	+++	++++	++++
7	+	++++	++++	++++	++++
8	+	+	+++	+	++
9	++	++++	++++	++++	++++
10	++	++	+++	+++	+++
11-20	++++	++++	++++	++++	++++
21-30	—	—	—	—	—

Experiment. Five antigen dilutions were prepared under similar conditions, 1 cc. antigen being mixed in each case with 1 cc. salt solution. Antigen dilution was used with a group of sera immediately after dilution. The remaining 4 antigen dilutions were used with the same sera 5, 10, 20 and 30 minutes, respectively, after their dilution. The tests were carried out by mixing 0.025 cc. of each of the antigen dilutions with 0.15 cc. serum. The tubes were

shaken 2 minutes in the usual manner, 0.5 cc. salt solution added to each tube and the results read and recorded.

It was observed that the effect of age of the antigen dilutions was negligible in the case of the strongly positive sera, i.e., sera which gave four plus reactions in each of the 3 tubes in the routine test. In the case of weaker sera, however, there was a tendency towards more sensitive precipitation reactions after the antigen dilution had been permitted to stand for some minutes. The greater fluctuations were found during the first 10 minutes after diluting the antigen. Table 12 gives the results with 30 different sera.

Serum 8 in table 12 is the only one which gave an anomalous reaction. All the remaining sera gave increasingly stronger reactions with age. On the basis of these findings an antigen dilution is permitted to stand 10 minutes before its use in the tests.

CHAPTER VI

QUANTITATIVE RELATION BETWEEN SERUM AND ANTIGEN

One of the outstanding features of the author's precipitation test is that the reacting substances of the serum lend themselves easily to quantitative measurements. Each syphilitic serum appears to have a definite capacity for combining with antigen, this capacity being determined by the number of reacting elements present in the serum. This chapter will discuss the serum-antigen relations and the factors governing them.

QUANTITATIVE REQUIREMENTS FOR COMPLETE PRECIPITATION REACTION

It is generally believed in connection with the Wassermann test that, if a given serum fails to give a complement fixation reaction in the presence of a given amount of antigen, the serum lacks sufficient reacting elements. This precipitation test indicates, however, that in the reaction between syphilitic serum and antigen there are at least two factors which determine negative reactions—a lack of reacting substances in the serum and the use of an excessive amount of antigen. The quantitative nature of this latter factor is well illustrated when diluting a given serum in series and testing these dilutions with different amounts of antigen. Table 13 gives the findings with 2 different sera.

The results in this table clearly indicate that the reaction of a serum giving complete precipitation with certain amounts of antigen dilution is rendered negative by an excessive amount. In other words, precipitates result from the union of serum and antigen only when the reacting sub-

TABLE 13
Inhibition of precipitation by an excessive amount of antigen dilution

	SERIES 1	SERIES 2	SERIES 3	SERIES 4	SERIES 5	SERIES 6	SERIES 7	SERIES 8
Antigen dilution, cc.....	0.3	0.15	0.1	0.075	0.05	0.025	0.0125	0.0125
Serum, cc.....	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.3
Serum diluted with salt solution	Results							
1:1	++	+++	+++	+++	+++	+++	+++	+++
1:2	-	+++	+++	+++	+++	+++	+++	+++
1:4	-	++	+++	+++	+++	+++	+++	+++
1:6	-	-	++	+++	+++	+++	+++	+++
1:8	-	-	-	++	+++	+++	+++	+++
1:16	-	-	-	-	++	+++	+++	+++
1:32	-	-	-	-	-	++	+++	+++
1:64	-	-	-	-	-	-	++	++
1:1	-	-	+	++	++	++	++	++
1:2	-	-	-	+	++	++	++	++
1:4	-	-	-	-	++	++	++	++
1:6	-	-	-	-	-	++	++	++
1:8	-	-	-	-	-	-	++	++
1:16	-	-	-	-	-	-	-	++
1:32	-	-	-	-	-	-	-	++
1:64	-	-	-	-	-	-	-	+
								-

stances in the antigen are in proper ratio with those in the serum. If the reacting elements of the antigen are in excess of those in the serum, negative results are obtained.

It appears also from the findings in table 13 that a complete precipitation reaction depends not so much on the quantities of serum and antigen dilution employed as on the proportions of these ingredients used. To what degree the same proportions of serum and antigen dilution give identical results without reference to the quantities employed is best illustrated by the results recorded in table 14 with 5 different sera. A serum-antigen proportion of 1:2, for example, gives a doubtful ($\pm$) reaction whether the quantities are 1.5 and 0.75 cc. or 0.15 and 0.075 cc. of serum and antigen dilution respectively.

THE SERUM-ANTIGEN AMOUNTS IN ROUTINE TESTS

In choosing 3 serum-antigen proportions in our routine test we were guided largely by practical considerations. Five or more proportions of serum and antigen dilution would be likely to give results of greater quantitative value to the clinician. It seemed wiser, however, to limit the routine test to 3 tubes and develop a special quantitative procedure applicable to the more strongly reacting sera.

The use of 3:1, 6:1 and 12:1 proportions of serum and antigen dilution was adopted after experiments indicated that the average precipitation results obtained with these proportions were specific. As might be expected, serum-antigen proportions approaching these, such as 4:1, 8:1 and 12:1, give similar results. It was further desired to limit the total amount of serum employed in the test to 0.5 cc. On this basis, 0.15 cc. amounts of serum were chosen for each of the 3 proportions. These serum amounts in turn necessitated the employment of 0.05, 0.025 and 0.0125 cc. of antigen dilution, respectively.

Since maximum precipitation in serum-antigen mixtures is obtained when the number of reacting elements of the serum and antigen approach one another, it is evident that sera containing a small number of reacting elements and giving a weak reaction in a serum-antigen proportion of 12:1 would give stronger reactions with proportionately lesser amounts of antigen. This is verified experimentally as shown in table 13. The last column in this table, where the serum-antigen proportion is 24:1, gives the most sensitive reactions. It seemed, however, that in the development of a practical test the aim should not be to render it as sensitive as possible. It is difficult to draw sharp lines of demarcation in biological tests. A test, in our opinion, capable of detecting a minute amount of specific reacting substance may also, even if infrequently, be capable of giving false reactions. On the basis of this consideration it seemed best to limit the serum-antigen proportion in the routine test to 12:1, after many thousand examinations indicated that the precipitation results in this proportion were specific for syphilis.

DIRECT DETERMINATION OF NUMBER OF REACTING SUBSTANCES IN SYPHILITIC SERA

Having discussed the quantitative requirements for complete precipitation reactions, we are ready to consider the numerical determination of the reacting substances in syphilitic sera. This, as is well known, has been attempted by means of the Wassermann test. The results, however, have been largely unsuccessful. The reason for this, in our opinion, is the fact that in complement fixation the intensity of the serum-antigen reactions is measured indirectly by complement binding. Knowing the variable nature of complement as well as of the other components of the hemolytic system, it seems apparent that an indicator

based on the disappearance of complement would interfere with quantitative measurements of the reacting substances in the sera.

In the precipitation test, however, the measure of the degree of the serum-antigen reactions is carried out directly by observing the intensity of precipitation. It was believed, therefore, that the test under discussion should adapt itself readily to quantitative measurements. It was further believed that the rapidity of the precipitation reactions inherent in the test might simplify such measurements by more clearly defining the end points. Although prolonged incubation would not prevent such quantitative determination, it would tend to equalize borderline reactions and thus interfere with the sharp distinctions between the weaker precipitation findings.

In attempting to determine to what degree the reacting substances in syphilitic sera may be numerically determined, it was first necessary to choose some unit of measurement. Since 0.15 cc. of serum and 0.0125 cc. of antigen dilution represent the smallest quantities used in the routine test, they were adopted in the derivation of the unit of precipitation reaction. The quantities are arbitrary—chosen merely to facilitate correlation with the regular test—the proportion of serum to antigen dilution being the important factor.

If 0.15 cc. undiluted serum mixed with 0.0125 cc. of antigen dilution results in a precipitation reaction of one plus, the serum may be considered to contain one unit of reacting substance. Due, however, to the fact that a one plus represents an incomplete precipitation reaction and carries comparatively little significance, it seemed best to consider only a complete or four plus reaction. Thus, when 0.15 cc. of serum represents the minimum amount giving a four plus reaction with 0.0125 cc. antigen dilution it is inter-

preted as a four unit reaction. To state this in outline form:

0.15 cc. undiluted serum
0.0125 cc. antigen dilution

Complete or four plus (+ + + +) reaction = four unit reaction

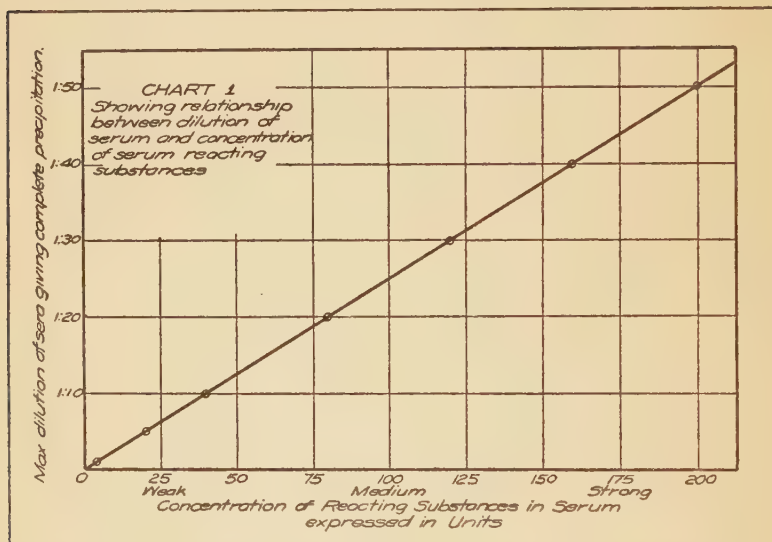
When attempting to ascertain the potency of a given serum on the basis of this unit system, two possibilities presented themselves: To employ 0.15 cc. quantities of undiluted serum with proportionately increasing quantities of the antigen dilution, or to employ a constant quantity of the latter, 0.0125 cc. and resort to proportional dilutions of serum. The latter was chosen because of simplicity and also in order to reduce the amount of serum required for a quantitative determination.

Experiment. Ten syphilitic sera from cases in varying stages of treatment were each diluted with salt solution in the following proportions: 1:1, 1:5, 1:10, 1:20 1:30, 1:40, 1:50, 1:60, 1:80, and 1:100, respectively. Each of these serum dilutions in 0.15 cc. amounts was mixed with the standard amount of antigen dilution, 0.0125 cc. The racks were shaken 2 minutes and the results read after each tube received 0.5 cc. salt solution as in the regular test. This experiment was then repeated daily for eight days using 5 sera of unselected cases undergoing treatment. Table 15 gives the results obtained with the 60 sera examined.

It will be noted that only complete or four plus precipitation reactions are recorded in this table. This is done in order to avoid confusion when calculating the number of reacting substances in a given serum on the basis of this unit system. Thus, the sera giving negative reactions when diluted 1:5 (table 15) contain 4 reacting units each, whereas those which give complete precipitation reactions,

when diluted 1:40 with salt solution, contain 40 times 4 or 160 reacting units.

When employing the maximum dilution of serum giving a complete or four plus reaction in 0.15 cc. amounts (table 15) as an ordinate and the concentration of reacting substances expressed in reacting units as an abscissa, the direct relationship between serum dilution and antibody concentration may be readily expressed by means of a curve



(Chart 1). This relationship can also be expressed as follows: Let S = the potency of serum expressed in reacting units and C = the concentration of reacting substances in serum. Then S varies directly as C or $S = kC$ where k is a constant. If D represents the maximum dilution of a serum giving a complete precipitation reaction, then the potency of a given serum is equal to four times the dilution or $4D = S$.

ABSORPTION OF SERUM REACTING SUBSTANCES WITH
ANTIGEN

The fact that each syphilitic serum possesses a definite capacity for combining with antigen—depending on the number of reacting substances present—can also be readily demonstrated by simple absorption experiments. Thus, to a given amount of syphilitic serum is added a measured amount of standard dilution of antigen. A precipitate is immediately formed. After removing the precipitate by centrifugation and adding another measured amount of antigen to the supernatant fluid, a precipitate may or may not be formed, depending on whether the first amount of antigen combines with or absorbs all the reacting substances. One may repeatedly obtain fresh precipitates with new amounts of antigen in the case of strongly positive sera, whereas in weak sera a comparatively small amount of antigen usually absorbs all the reacting substances present, and further addition of antigen gives negative results.

These absorption experiments give results similar to those of the quantitative dilution tests just described, the difficulty with the former being the requirement of a considerable amount of time and labor in carrying out a single determination. The absorption method is also limited in its application to unusually potent sera. The reason for this is that most sera have to be diluted with several amounts of salt solution before determining the number of reacting substances by means of antigen absorption. This is to enable one to throw down the precipitate by centrifugation. When a given amount of antigen dilution is added to undiluted serum and a precipitate is formed, centrifugation, in most cases, does not throw down but clumps the precipitate and leaves it in suspension.

EFFECT OF EXCESSIVE AMOUNTS OF SERUM

The inhibitory effect of an excessive amount of antigen dilution has been fully considered. With regard to serum, the addition of an excessive amount, such as one hundred parts of serum to one of antigen dilution, while not inhibitory to precipitation, affects the character of the precipitate. To a certain degree, a similar influence is exerted by an excessive amount of serum as by an excessive amount of salt solution. Thus, while 3, 6 or 12 parts of serum to 1 of antigen dilution may produce heavy precipitates, 50 and 100 parts of serum to 1 of antigen dilution produces but fine precipitates. Furthermore, while the lesser serum amounts produce heavy clumps after perhaps an hour's incubation, the larger serum amounts do not produce clumps of the same character even after 24 hours' incubation.

AN ANOMALOUS REACTION

When employing 3:1, 6:1 and 12:1 proportions of serum to antigen dilution, weakly reacting syphilitic sera give stronger reactions with the lesser amounts of antigen dilution. It was observed in our early studies with this method that some sera gave stronger reactions with the larger amounts and negative reactions with the lesser amounts of antigen dilution. This finding for a time appeared so constant, particularly in connection with sera from latent lues, that in a preliminary communication on this method we suggested that this reaction might possibly be indicative of latent lues. Strange as it may seem, however, when using our newly standardized antigen with which we have made about 25,000 determinations, the reactions are uniformly stronger with the lesser amounts of antigen dilution. The fact that this anomalous reaction was observed only infrequently in our earlier studies and not at all in connection with our newly prepared antigen suggests that we might

have dealt with a peculiar serum or antigen condition and not necessarily with a given clinical entity.

It would appear, on the other hand, that a similar anomalous reaction is also observed in complement fixation tests in syphilis. Thus, in tests employing gradations of serum with the same amount of antigen, it infrequently happens that the larger amount of serum gives a negative reaction while a lesser amount gives a positive reaction. That this is not due to the presence of natural amboceptor is indicated by the fact that we have observed such reactions with sera from which the natural amboceptor was entirely removed. This phenomenon may be related to that described by Coca and L'Esperance (22). When employing raw sera with an actone-insoluble antigen in complement fixation tests, these observers noted definite reacting zones where positive reactions were obtained, the reactions not being dependent upon the larger amounts of sera.

Should certain antigens give these anomalous reactions, no practical difficulty would present itself, since our three antigen proportions used in the test would cover just such fluctuations.

IMPORTANCE OF QUANTITATIVE MEASUREMENTS OF SERUM SUBSTANCES IN SYPHILIS

The quantitative measurement of reacting substances in syphilitic sera, possible by this precipitation method, throws light on several serological problems associated both with the Wassermann test and the various precipitation tests. In the first place our findings indicate that a precipitation test based on one serum-antigen proportion such as our earlier procedure as well as some of the tests proposed by different authors can not reach the highest practical value. Even for a purely qualitative test, a single serum-antigen proportion is likely to give good results only by employing the smallest amount of antigen possible. Any amount greater than the

minimum would be likely to render some weak reactions negative.

With regard to the Wassermann test, it is well known that some clinicians do not consider weak reactions such as + + or + as indicators of the degree of syphilitic involvement. Wile (23), for example, has for a long time contended that a weak Wassermann reaction may have the same significance as a four plus reaction and, if during a given course of treatment a syphilitic patient gives a two plus reaction, it does not necessarily mean clinical improvement, since the same patient is likely to give a four plus reaction when the course of treatment is completed. Judged from our present studies, it appears that this view may be correct in many cases. A weak Wassermann reaction may not necessarily be related to the number of reacting elements in the patient's blood, but rather to a disturbance in the correct serum-antigen balance in the test. It is not easy to determine the validity of such a contention when dealing with complement amboceptor and corpuscles aside from the serum and antigen. The indirect method of determining the degree of reaction between serum and antigen in the Wassermann test renders the correct quantitative measurements of reacting substances in sera extremely difficult. The precipitation test, on the other hand, being free from the complications inherent in complement fixation, should be capable of giving correct quantitative determinations of the number of reacting elements in sera and, if this number is related to clinical involvement, the test should prove an important means of measuring such involvement.

It is interesting to note in this connection that the amount of antigen usually employed in the Wassermann test is still largely arbitrary. It is, for example, believed by many workers (Ottenberg (24), Kolmer (25)) that, when employing a fixed amount of complement, the larger the number of antigen units employed in a given test, provided the number

is neither anticomplementary nor hemolytic, the stronger the complement fixation reaction. Antigen studies carried out by Johnson with the writer (26) in this laboratory indicated that too large a number of antigenic units with a standard amount of complement was capable of rendering weak reactions negative. Thus, with certain syphilitic sera, where 0.1 cc. antigen-saline mixture containing 2 to 3 antigenic units gave a +++ or even ++++ reaction, 0.3 cc. of the same antigen mixture containing about 8 antigenic units gave a + or negative reaction. These observations with the Wassermann test appear to be corroborated by our findings with the precipitation test which also indicate that an excess of antigen is to be avoided, particularly with weaker sera.

CHAPTER VII

FACTORS AFFECTING SENSITIVENESS AND UNIFORMITY OF ANTIGEN

Although the antigen employed in the author's test gave results of relative uniformity, it was frequently observed that with isolated sera giving weak serologic reactions certain antigens gave results of lesser sensitiveness than others. Studies were thereupon undertaken with a view toward so standardizing the method for antigen preparation as to assure a high degree of uniformity. The aim was to produce an antigen not of the highest sensitiveness but rather of a relatively high degree of sensitiveness combined with the utmost reliability. The problem was extensive because the factors governing three different phases in antigen preparation had to be determined: the preliminary extraction with ether, the alcohol extraction and the cholesterinization. It seemed best to present the complete studies on antigen standardization elsewhere and limit this discussion to the more essential phases of the problem.

PRELIMINARY EXTRACTION WITH ETHER

The purpose of the ether extraction is to remove ether soluble lipoids which interfere with the reaction. An antigen prepared by extracting powdered muscle directly with alcohol is capable of producing precipitates with syphilitic sera but does not approach in sensitiveness the antigen employed in the author's test. It will be recalled that the antigen used in this test, when diluted with approximately an equal amount of salt solution, gives a precipitate which is soluble in serum. A direct alcoholic extract antigen, on the other hand, when diluted with varying amounts

of salt solution, gives a precipitate which is stable and incapable of dissolving in serum.

The question that presented itself was: What should be the criterion of the ether extraction? In our early method of antigen preparation, ether extraction of the beef heart was continued until the ether possessed no coloring matter. The extractions were carried out at ordinary icebox temperature at 24 hour intervals and, as a rule, four liberal amounts of ether were sufficient.

There were two inherent weaknesses in this method. Fluctuations in icebox temperature which frequently could not be overcome affected the extraction. It was difficult, furthermore, to judge the endpoint. Some workers would continue the extraction until the supernatant ether was entirely color free. Others would consider the extraction completed when there was a trace of coloring matter in the ether, and still others when there was considerably more than a trace of coloring matter present. These variations, we believed, affected the final product and interfered with uniformity.

In attempting to determine the reason why a given lot of antigen, although prepared in the regular manner, was somewhat less sensitive than usual, it appeared that over-extraction with ether was a possible factor. The following preliminary experiment was thereupon carried out: Five cubic centimeter quantities of this less sensitive antigen were added to a series of 5 tubes and 0.01, 0.025, 0.05 and 0.1 cc. quantities of a concentrated ether extract of powdered beef heart were respectively added to the 5 tubes and thoroughly mixed. One cubic centimeter quantities of each of these modified antigens were now mixed with equal amounts of salt solution and the solubility of the resulting precipitates tested in salt solution and negative serum. It was found that all precipitates were soluble except that formed in the antigen to which was added the 0.1 cc. amount

ether extract. Further observations soon showed that 0.05 cc. of the ether extract added to 5 cc. of the antigen represented the maximum amount which did not interfere with the use of the antigen in the test. The next step was to test the comparative sensitiveness with syphilitic sera of the original antigen and the same antigen containing 1 per cent of ether extract—0.05 cc. per 5 cc. of antigen. It was observed that the latter antigen was considerably more sensitive than the original one, thus indicating that fluctuations in sensitiveness heretofore observed in antigens might have been due to fluctuations in the ether extractions.

On repeating the above experiment with antigens of varying sensitiveness, it was observed that the amount of ether extract that could be added to a given antigen without rendering it unfit for use in the test was indirectly proportional to the sensitiveness of the antigen. Thus, to an antigen of high sensitiveness only 0.1 per cent of the ether extract could be added with safety; to an antigen of somewhat lesser sensitiveness, perhaps 0.5 per cent could be added; while to an antigen of low sensitiveness, as much as 1 per cent of the ether extract could be added. These findings suggested that it was not desirable to completely remove the ether extractives from the powdered beef heart.

The following more quantitative experiment was carried out to further prove that excessive extraction with ether reduces the sensitiveness of an antigen and that the lack of sensitiveness may be counteracted by the addition of a definite amount of ether extractives according to titration.

Experiment. Twenty-five grams powdered beef heart were placed in a 250 cc. Erlenmeyer flask and 150 cc. ether added. Extraction was carried out for 2 days at room temperature with frequent shaking. After this period, the ether was filtered off, the moist heart muscle replaced in the flask, and extracted again for a 2-day period in 100 cc.

fresh ether. Three further extractions at 2 day intervals were carried out, each with 100 cc. quantities of fresh ether. After a total of 5 separate extractions over a period of 10 days—thus insuring an excessive extraction with ether—the ether was filtered off and the residue extracted with 5 times the amount of 95 per cent alcohol for three days at room temperature.

The total amount of ether used in the ether extractions was collected and concentrated by evaporation to 50 cc. Varying amounts of this concentrated ether extract were added to the alcoholic extract in the following proportions:

	TUBE 1	TUBE 2	TUBE 3	TUBE 4
Alcohol extract + concentrated ether extract, cc....	5 + 0.025	5 + 0.05	5 + 0.075	5 + 0.1

One cubic centimeter amounts of each of these modified alcoholic extracts were now mixed with equal amounts of salt solution and the resulting precipitates tested for solubility in salt solution. It was found that only the precipitates resulting from the dilution of the extracts in tubes 1 and 2 possessed this property. It was thus evident that 0.05 cc. or 1 per cent of the concentrated ether extract represented approximately the largest amount which could be added to 5 cc. of the alcoholic extract without interfering with its use in the test.

When testing the comparative sensitiveness with serum of the original alcoholic extract and the same extract plus 1 per cent of the concentrated ether extract, it was again observed that the modified alcoholic extract was considerably more sensitive than the original one.

The results of this as well as of the previous experiment indicated that the titration of different alcoholic extracts with varying amounts of ether extract of definite concentration might furnish a means of standardizing antigens for

this test. This titration method appeared practicable for a time and various stock solutions of alcoholic extract were rendered sensitive to a highly uniform degree by adding the maximum amount of concentrated ether extract that they were capable of holding. It subsequently appeared, however, that this titration procedure might be unnecessary if the ether extraction of the beef heart could be so limited as to secure removal of only the required amount of extractives.

In attempting to thus control the ether extraction, it was necessary, in order to assure uniformity, to standardize the conditions of extraction. As a first step, a 25 gm. quantity of beef heart, to be extracted in a 250 cc. Erlenmeyer flask, was chosen as the unit of extraction. As a further step in producing standard conditions, it was desirable to eliminate the use of icebox temperature from the ether extractions, as fluctuations in icebox temperature in different laboratories were likely to lead to variations in antigen sensitiveness. In judging the endpoint of ether extraction, furthermore, the criterion was to be not freedom from coloring matter in the supernatant ether but rather the character of the final product as determined by special experiments with sera. It should be stated in this connection that in all the tests with sera by which we judged the endpoint of the ether extraction—as well as of the alcohol extractions to be discussed presently—cholesterin was added to the antigen in a concentration of 0.6 per cent.

In order to prevent over-extraction with ether, it was thought that 1 hour extraction periods at room temperature might be substituted for the usual 24 hour periods in the icebox. The following 5 antigens were thereupon prepared in order to determine the number of such extraction periods which might give desired results. The alcohol extraction was carried out in each case for 3 days at room temperature, it having been observed that this temperature gave slightly

superior antigens compared with extraction for 10 days in the icebox.

Antigen 1: 25 grams beef heart were extracted with 100 cc. ether for 1 hour at room temperature. Ether was filtered off and the powder freed from ether odor. Alcohol extraction (5 cc. per gram of beef heart) was carried out for 3 days at room temperature.

Antigen 2: 25 grams beef heart were extracted with 100 cc. ether for 1 hour at room temperature. Ether was filtered off and replaced with 75 cc. fresh ether. Extraction was continued for 1 hour at room temperature. Alcohol extraction was carried out as above.

Antigen 3: 25 grams of beef heart were extracted during three intervals of 1 hour each with 100, 75 and 75 cc. quantities of ether, respectively. Dried beef heart was subsequently extracted with alcohol as above.

Antigen 4: 25 grams of powdered beef heart were extracted during four intervals of 1 hour each with 100, 75, 75 and 75 cc. ether, respectively. Alcohol extraction was carried out as above.

Antigen 5: 25 grams of powdered beef heart were extracted during five intervals of 1 hour each with 100, 75, 75, 75 and 75 cc. ether, respectively. Alcohol extraction was completed as above.

Each of the above antigens was cholesterinized by adding 6 mgm. cholesterin per cubic centimeter.

One cubic centimeter amounts of each of these 5 antigens were mixed in the usual manner with equal amounts of salt solution and the precipitates formed in each case tested for solubility in salt solution. It was found that antigens 1, 2 and 3 gave insoluble precipitates and therefore could not be used in the test. Antigens 4 and 5 produced precipitates which readily dissolved in salt solution and antigen 4, representing the minimum number of ether extractions, was chosen for special study with regard to sensitiveness and specificity.

Several thousand routine examinations in this laboratory with antigen 4 indicated that with isolated sera from treated cases it was not quite as sensitive as desirable. It was thought that a further reduction in the ether extractions might bring the antigen up to the desired degree of sensitiveness. We thereupon decided to substitute 4 minimum extraction periods lasting 5 minutes each for the 1 hour periods. This, it was believed, might indicate how far the ether extraction periods might be reduced and still obtain antigens which could be used in the test.

The antigens obtained with the four 5 minute ether extractions periods were highly sensitive. The only difficulty was that the negative reactions were not sufficiently clear-cut in character—the tubes which contained the larger amounts of antigen showing slight clouding although apparently free from precipitates.

Further studies soon indicated that 4 ether extraction periods lasting 10 minutes each produced alcoholic extracts approaching the desired degree of sensitiveness and giving clear-cut negative reactions. Before adopting this ether extraction method in the standard procedure of antigen preparation, it was desired to determine whether this method actually represented the minimum removal of ether extractives compatible with the test. To antigens prepared by utilizing this method of ether extraction, were thereupon added varying amounts of a concentrated solution of ether extractives. It was observed that 0.1 per cent (0.005 cc. per 5 cc. of antigen) of this solution was the maximum that could be added without rendering these antigens unfit for use in the test. This indicated that the four 10 minute extraction periods removed an amount of ether extractives approaching the minimum.

It was further desired to test the comparative sensitiveness with sera from unselected cases undergoing antisyphilitic treatment of an antigen prepared by utilizing the 10 minute

TABLE 16
Relation of ether extraction to sensitiveness of antigens in tests with sera from cases undergoing treatment

Ether extraction.....	ANTIGEN 1				ANTIGEN 2				ANTIGEN 3			
	Excessive				Excessive				*Standard			
	0				1				0			
Serum:antigen dilution.....	3:1	6:1	12:1		3:1	6:1	12:1		3:1	6:1	12:1	
Serum number	Results											
1	++	++	++	++	++	++	++	++	++	++	++	++
2	+	+	+	+	+	+	+	+	+	+	+	+
3	++	++	++	++	++	++	++	++	++	++	++	++
4	++	++	++	++	++	++	++	++	++	++	++	++
5	++	++	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	++	++	++	++	++	++	++
7	++	++	++	++	++	++	++	++	++	++	++	++
8	++	++	++	++	++	++	++	++	++	++	++	++
9	++	++	++	++	++	++	++	++	++	++	++	++
10	++	++	++	++	++	++	++	++	++	++	++	++
11	++	++	++	++	++	++	++	++	++	++	++	++
12	++	++	++	++	++	++	++	++	++	++	++	++
13	++	++	++	++	++	++	++	++	++	++	++	++
14	++	++	++	++	++	++	++	++	++	++	++	++
15	++	++	++	++	++	++	++	++	++	++	++	++
16	++	++	++	++	++	++	++	++	++	++	++	++
17	++	++	++	++	++	++	++	++	++	++	++	++
18	++	++	++	++	++	++	++	++	++	++	++	++
19	++	++	++	++	++	++	++	++	++	++	++	++
20	++	++	++	++	++	++	++	++	++	++	++	++

* Four extraction periods of 10 minutes each using 100, 75, 75 and 75 cc. ether, respectively.

extraction periods, one in which the ether extraction was excessive and the same antigen plus 1 per cent ether extractives—this amount representing the maximum the antigen could hold and still be fit for use in the test. The alcohol extraction of each of these antigens was carried out under identical conditions for three days at room temperature, after which cholesterin was added in the proportion of 6 mgm. per cubic centimeter. Each antigen was diluted for the serum tests with an equal amount of salt solution. The results with 20 sera shown in table 16 are typical and indicate that antigen 3, in which the four 10 minute ether extraction periods were used, is comparable in sensitiveness with antigen 2, which was treated with a titrated amount of ether extractives. On the basis of these findings, an ether extraction consisting of four 10 minute periods, employing 100, 75, 75 and 75 cc. of ether, respectively, was accepted as a standard procedure in the preparation of antigen.

EXTRACTION WITH ALCOHOL

As already indicated, a 3 day alcohol extraction at 21°C. was employed in connection with the standardization of the ether extraction—the desirability of this alcohol extraction method having been suggested by preliminary experiments. It was now important to study further the extent to which different temperatures and periods of the alcohol extraction—employing our newly standardized ether extraction method as a constant—might modify the final product. Preliminary extractions at different temperatures and periods soon indicated that these could be varied considerably without affecting the behavior of the final product with strongly positive sera; only with comparatively weak sera could the varying degrees of sensitiveness be measured.

In attempting to determine the optimum temperature for alcoholic extraction, comparative tests with weaker

sera indicated that antigens prepared by extracting at 37° and 56°C. were somewhat more sensitive than those extracted at 21°C., and the latter, in turn, slightly more sensitive than those extracted at 10°C.

The antigens prepared at 37° and 56°C. were found to possess two practical difficulties. The alcoholic extracts became turbid at room temperature with subsequent precipitation of lipoids soluble only at temperatures higher than 21°C. After filtering out this precipitate, there was a further gradual sedimentation of lipoids extending over a period of some weeks. This was thought likely to interfere with the uniformity of the extract. Another practical difficulty with antigens extracted at 37° or 56°C. was that the serum-antigen mixtures in the case of some negative sera appeared somewhat cloudy.

About 21°C., or room temperature, seemed the most practical extraction temperature. The optimum period of extraction was still to be determined. Extraction periods varying from 1 hour to 10 days were found to produce antigens approaching each other in sensitiveness. When taking into consideration, however, the appearance of the negative reactions—in addition to sensitiveness and specificity of these various antigens as indicated by comparative routine tests extending over a period of several months—a 3 day alcohol extraction at room temperature was chosen as a part of the standard method for antigen preparation.

CHOLESTERINIZATION OF ALCOHOLIC EXTRACT

A cholesterin-free antigen, when titrated according to the method outlined in a following chapter, gives precipitation results of considerable sensitiveness. It is our opinion, based on preliminary experiments, that a cholesterin-free antigen may be so modified as to give practically the same results as a cholesterinized antigen,

except that it would add considerable manipulation to the procedure. For a practical test it appeared best to resort to the use of cholesterin—in such concentration as to assure a high degree of sensitiveness without interfering with specificity.

As already indicated, 0.6 per cent cholesterin was employed in all comparative studies on the standardization of antigen for this test. The substitution of this amount of cholesterin for 0.4 per cent, employed in the earlier method, was based upon clinical studies as well as comparative tests with the Wassermann as employed in this laboratory. The rôle of cholesterin in this test is now being investigated. Table 17 gives the results obtained with antigens containing varying amounts of cholesterin in a group of sera from cases of syphilis undergoing treatment. The tests were limited to 3:1 and 12:1 serum-antigen proportions because of insufficient serum. When one considers that the final result in our routine test is the average finding of the three tubes, it is evident that the difference in sensitiveness between the cholesterin-free and the various cholesterinized antigens is relatively small.

CHAPTER VIII

FACTORS AFFECTING SERUM

There are several factors which affect the behavior of serum in the author's test. This chapter will consider the effect of heating serum at 56°C. and at higher temperatures and also several other phases in connection with serum.

UNHEATED VERSUS HEATED SERA

It was early observed that for dependable results the test required the heating of sera at 56°C. This seemed of interest since we were accustomed to think of inactivation of sera in complement fixation tests as a means of destroying native complement. A comparative study of the sensitiveness of the test with unheated and heated sera was carried out with 544 specimens. A regular test was carried out with each serum both before and after heating for 30 minutes at 56°C. A summary of the results is presented in table 18. Of 177 sera which gave strongly positive reactions after inactivation, 48 were strongly positive, 31 weakly positive and 98 negative in an unheated state.

These findings suggested that some generally accepted views regarding the inactivation of sera for the Wassermann test were not entirely correct. In the heating of syphilitic sera for complement fixation tests, various workers have indicated that, in addition to the destruction of complement, there is considerable destruction of the specific reacting substances. With a view of minimizing the destruction of these substances, many have reduced the heating period of sera from 30 to 10 or 15 minutes. Quantitative heating experiments of sera carried out in this laboratory (27, 28)

in connection with the Wassermann test showed that many sera gave stronger complement fixation reactions after 30 than after 10 or 15 minutes' heating, particularly when prolonged fixation at icebox temperature was employed. We believed at that time that the weaker reactions in the Wassermann test following 30 minutes' heating at 56°C., observed by some workers, were not due to the destruction of the reacting substances by heat but rather to factors unrelated to serum, as for example, the kind of antigen used in the test and the length of the fixation period. The fact that this precipitation test required the heating of serum at 56°C. for correct results appeared to corroborate our experimental findings in

TABLE 18

Comparative findings with inactivated sera and the same sera before inactivation

NUMBER OF SPECIMENS	INACTIVATED			NOT INACTIVATED		
	++++ or +++	++ or +	—	++++ or +++	++ or +	—
177	177			48	31	98
19		19				19
348			348			348

connection with complement fixation and indicated that we were not dealing in the heating process simply with the destruction of complement and specific reacting substances in the serum, but with other factors which markedly affected the reactions of syphilitic serum with antigen.

It seemed possible that the difference in sensitiveness between raw and heated sera was due to slight changes in the H ion concentration brought about by inactivation. Marshall, in this laboratory, investigated the difference in the H ion concentration of unheated and heated sera with special reference to their behavior in the author's test. Measurements were made colorimetrically, using phenol-sulphonphthalein as an indicator and standards made

from electrometrically controlled buffer solutions. Of 64 positive sera (26 unheated, 38 heated) and 186 negative sera (74 unheated; 112 heated), the average of the differences between unheated and heated sera, and between positive and negative sera, was approximately the same as between the individual sera of the same class. To illustrate: The average difference between unheated and heated sera was approximately pH 0.05 for the positive sera, while most of the heated sera showed a slightly higher pH. The majority of the individual sera, however, showed a greater difference than pH 0.05, and in some cases unheated sera showed the slightly higher pH. It thus appeared that there was no consistent variation in the H ion concentration between unheated and heated sera (including both positive and negative) under conditions in this laboratory where the specimens are sent in through the mails.

PERIOD OF INACTIVATION

In attempting to determine the period of inactivation of sera at 56°C. which would give optimum precipitation reactions, the following experiment was carried out.

Experiment. Unheated syphilitic sera and some non-syphilitic sera as controls were each divided into 6 parts. One part of each of the sera was allowed to remain unheated while the remaining 5 parts were heated at 56°C. for 5, 10, 20, 30 and 60 minutes, respectively. All these parts were tested in 0.15 cc. amounts with 0.025 cc. quantities of standard antigen dilution. The tests were shaken for 2 minutes and the results read after the addition of 0.5 cc. salt solution to each tube. The results are indicated in table 19.

It is evident from this table that the more potent the serum the shorter the inactivation period required. The very strongly positive sera apparently did not require inactivation. Sera of somewhat lesser potency required

no more than 5 minutes of inactivation, while still weaker sera apparently required a minimum of 30 minutes' heating. Of especial interest is the finding that the reactions after 30 minutes' heating were considerably stronger than after 20 minutes' and that after 60 minutes' some reactions

TABLE 19

Effect of various periods of heating at 56°C. on sera of different potency

NUMBER OF SERA IN GROUP	PERIODS OF HEATING AT 56°C.					
	None	5 minutes	10 minutes	20 minutes	30 minutes	60 minutes
	Results					
10	++++	++++	++++	++++	++++	++++
11	—	++++	++++	++++	++++	++++
3	+	+	+++	++++	++++	++++
2	—	++	++++	++++	++++	++++
7	—	+	++	++++	++++	++++
1	—	—	++++	++++	++++	++++
1	—	—	+	++++	++++	++++
1	—	—	+	++	++++	++++
1	—	+	++	+++	+++	+++
1	—	+	++	+++	++	++
2	—	+	++	+++	++++	++++
1	—	—	—	++	+++	++++
1	—	—	—	++	++++	++++
1	—	—	—	++	+++	+++
1	—	—	—	+	+++	+++
5	—	—	—	+	++	++
1	—	—	—	+	+	+++
1	—	—	—	—	++	+++
1	—	—	+	+	+	+
1	—	—	—	—	+	+
3	—	—	—	—	—	—

were slightly stronger than after 30 minutes' heating. This brought up the problem whether extending the heating period beyond 60 minutes might not in some cases give still stronger reactions. The following experiment was thereupon carried out.

Experiment. A number of positive sera were each divided into four parts. One part of each serum was

heated for 30 minutes at 56°C., the other part for 90, the third for 120 and the fourth part for 180 minutes. Each

TABLE 20
Effect of prolonged heating of sera at 56°C.

NUMBER OF SERA	HEATING PERIODS AT 56°C.			
	30 minutes	90 minutes	120 minutes	180 minutes
	Results			
22	++++	++++	++++	++++
1	++++	++++	+	±
2	++	+++	+++	+++
1	++	++	++	+++
1	++	++	++	±
1	++	++	++	++
1	+	+	+	+
1	+	+	±	±
1	+	±	±	-
1	±	±	-	-
18	-	-	-	-

TABLE 21
Effect of heating sera at a temperature higher than 56°C.

NUMBER OF SERA	HEATING PERIOD			
	At 56°C. for 30 minutes	At 62°C. following 30 minutes at 56°C.		
		30 minutes	60 minutes	120 minutes
Results				
5	++++	++++	++++	++++
3	++++	++++	++++	+++
4	++++	++++	++++	+
3	++++	++++	++	+
1	++++	++++	—	—
1	++++	+++	—	—
3	++++	++	—	—
5	++++	+	—	—
6	+	+	—	—
2	+	—	—	—
17	—	—	—	—

part in a 0.15 cc. amount was mixed with 0.025 cc. antigen dilution and the tests completed in the usual manner.

The results of this experiment are recorded in table 20. It is evident that even three hours' heating at 56°C. had

TABLE 22
Effect of re-heating sera 24 and 48 hours after inactivation

NUMBER OF SERA	HEATING PERIOD AT 56°C. 30 MINUTES	RE-HEATING PERIODS AT 56°C.			
		None	5 minutes	10 minutes	15 minutes
		Results			
24 hours after inactivation*					
13	++++	++++	++++	++++	++++
3	+++	+++	+++	+++	+++
2	++++	+++	++++	++++	++++
1	+++	—	±	++	++
1	++	+	+	++	++
1	++	—	±	+	++
1	++	—	+	+	+
1	+	+	++	+++	+++
1	+	—	±	±	±
2	±	—	±	±	±
1	±	—	—	+	±
1	±	—	—	±	±
48 hours after inactivation*					
24	++++	++++	++++	++++	++++
1	++++	+++	++++	++++	++++
1	+++	++	+++	+++	+++
1	+++	±	+	++	++
1	++	+	+	+	+
1	+	++	+++	+++	+++
1	+	±	+	++	++
1	+	±	±	+	+
1	+	—	±	+	+
1	+	—	±	±	++
1	±	±	+	+	++
1	±	—	±	+	++
1	±	—	±	+	+
1	±	—	++	++	++
1	±	—	±	±	±
1	±	±	±	±	±

* Sera kept in icebox after inactivation.

but little destructive effect on the reacting substances of the various sera tested.

It was of interest to carry out a similar experiment employing a temperature higher than 56°C. The following temperatures and periods were used: 30 minutes at 56°C.; 30, 60 and 120 minutes at 62°C. In studies on the effect of heating sera for 20 minutes at 62°C. in the Wassermann test, marked weakening of the reactions was observed. It was therefore desired to find to what extent the heating of sera at this temperature would give comparable results.

Table 21 records the findings with this experiment. While 62°C. was apparently destructive to the reacting substances in syphilitic sera, prolonged heating at this temperature was apparently necessary in many cases to bring about this effect.

Still another problem presented itself in connection with the inactivation of sera for this test. It was necessary to determine if sera, after standing for a day or more in the icebox, should be reactivated before re-testing. The following experiment was thereupon carried out.

Experiment. A group of sera was inactivated for 30 minutes at 56°C. and examinations made by employing 0.15 cc. quantities with 0.025 cc. amounts of antigen dilution. The sera were then placed in the icebox and some re-examined after 24 and others after 48 hours. These re-examinations were made in each case without reheating the sera and after 5, 10 and 15 minutes' reheating at 56°C., respectively. Table 22 shows that the results in some cases became weaker after the sera had remained in the icebox for 24 or 48 hours, but after reheating these specimens for 10 or 15 minutes the results approached those obtained before placing the sera in the icebox.

EFFECT OF CONTAMINATION AND AGE ON SERA

We were led to investigate this problem because of its practical importance. It was observed again and again that blood specimens or spinal fluids reaching this laboratory

TABLE 23
Effect of age and contamination of serum upon the Kahn and Wassermann tests
 Negative sera

	TIME OF INCUBATION AFTER FIRST EXAMINATION								TOTALS	PER CENT		
	3 days		5 days		7 days		10 days					
	26	61	31	24	142							
Total number of sera.....												
	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test		
Sera remaining negative.....	25	21	58	30	29	22	17	10	129	83	90.8	58.5
Sera unfit for Kahn or anticomplementary in Wassermann.....	1	5	1	28	2	9	6	12	10	54	7.1	38.0
Sera changing to positive.....	0	0	2	3	0	0	1	2	3	5	2.1	3.5

Positive sera

	Positive sera								TOTALS	PER CENT		
	9		18		16		9					
	52	52	52	52	52	52	52	52				
Total number of sera.....												
	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test	Kahn test	Wasser-mann test
Sera remaining positive.....	7	2	8	5	14	10	6	4	35	21	67.3	40.4
Sera unfit for Kahn or anticomplementary in Wassermann.....	0	7	0	8	0	5	2	5	2	25	3.8	48.1
Sera changing to negative.....	2	0	10	5	2	1	1	0	15	6	28.8	11.5

in apparently contaminated condition and often giving anticomplementary reactions in the Wassermann test gave specific reactions in the author's test. This brought up the question, to what extent a serum may be contaminated and still give apparently correct precipitation results.

Miss Kendrick (29) in this laboratory, who carried out this investigation, inoculated a number of syphilitic and non-syphilitic sera with different organisms, such as *Staphylococcus aureus* and Gram-negative bacilli isolated from contaminated serum. Natural air contamination was permitted in other sera. These sera were incubated at 37°C. for 3, 5, 7, and 10 days, respectively. Wassermann and precipitation tests were made after these intervals. The results are illustrated in table 23.

Several sera became unfit for use in the author's test because of precipitates which could not be removed by centrifugation. As a whole, however, the effect of age and contamination on the precipitation test was far less marked than on the Wassermann test, which might perhaps have been expected due to nonspecific fixation of complement in the latter test.

It should be stated that the experiment recorded in this table was limited to unheated sera; that is, the inoculation of the various organisms were made in unheated sera, although the sera were in every case heated for 30 minutes at 56°C. before performing the complement fixation and precipitation tests.

A similar series of experiments was carried out with sera which were first heated at 56°C. for 30 minutes and then inoculated with various organisms. These sera were reheated, in every case, for 10 minutes before performing the tests. A total of 223 negative and 100 positive sera were employed. The effect on both tests was found to be far less marked than in the case of the series of unheated sera.

These experiments indicate that there is less danger in using old or contaminated sera in the precipitation test than in the Wassermann. For reliable results in the author's test, however, it is obviously necessary to have the serum clear and free from contamination.

ANTICOMPLEMENTARY SERA

As already indicated, the reactions of sera anticomplementary in the Wassermann test are apparently unaffected in the precipitation test. This is particularly illustrated in table 23. According to this table, of a total of 194 positive and negative sera, 79 became anticomplementary due to contamination and age. Of this number, 12 sera were unfit for use in the precipitation test, due to inability to render them free from particles. The remaining 67 sera, however, gave either positive or negative reactions entirely comparable to the original reactions given by the same sera before they became anticomplementary.

CHAPTER IX

SHAKING OR AGITATION

The first observations showing that the formation of precipitates was hastened by shaking the serum-antigen mixture was made in connection with our early precipitation studies. It was observed that the same sera tested by different workers gave results varying considerably in sensitiveness. An attempt to determine the reason for this variation led to the discovery that those workers who habitually shook the tests vigorously for 15 seconds or more after adding serum to antigen dilution obtained more sensitive results than those who shook the tests but slightly for a few seconds. Further observations led to the use of a 3 minute shaking period in the earlier procedure. With the employment of the standard antigen-saline dilution, the problem of shaking was reinvestigated.

EFFECT OF SHAKING ON SENSITIVENESS OF REACTIONS

When studying the effect of shaking on negative sera, it was soon observed that there was no tendency for the formation of non-specific precipitates. Shaking indeed showed a tendency towards clearing serum-antigen mixtures. This was observed particularly in connection with incubation experiments. Negative serum tests which showed doubtful precipitates after 1 to 4 hours' incubation were readily rendered clear by vigorous shaking for a few seconds.

With regard to positive sera, it was observed that those giving strong reactions did not require shaking. The reactions appeared to take place instantaneously on mixing these sera with antigen dilution. Weaker sera, however, required shaking for optimum reactions. This is illustrated by the following experiment.

Experiment. Sera giving weakly positive reactions and diluted strongly positive sera were chosen in this experiment. The tests were carried out with 0.025 cc. antigen dilution and 0.15 cc. serum. After adding the serum to the antigen dilution, the tests were in each case shaken for a uniform period of 10 seconds to assure thorough mixing of the ingredients. Four tests were run with each serum, employing shaking periods of 1, 2, 3 and 4 minutes, respectively. Each of the 4 tests was controlled by a similar test which was not shaken but allowed to remain at room temperature during the same period. After each shaking period, the tests and their controls were read following the addition of 0.5 cc. salt solution to each tube.

It was observed that the tests which were shaken gave decidedly stronger reactions than those which were not shaken. It was further observed that a 1 minute shaking period was sufficient to produce optimum precipitation reactions in a large number of the sera tested. After 2 minutes' shaking most of the tests showed maximum results. In a few of the sera, 3 and 4 minute shaking periods slightly increased the strength of the reactions. Table 24 gives the results of tests with 40 sera.

This preliminary experiment having established the importance of shaking and the fact that a 1 to 3 minute period is ample to bring about maximum precipitation, the following experiment was carried out to determine the effect of various shaking periods on the routine test.

Experiment. Ten syphilitic sera of varying serologic potency were employed in this experiment. The standard antigen dilution was employed in 0.05, 0.025 and 0.0125 cc. quantities and the serum in 0.15 cc. amounts. After adding the serum to the antigen dilution, each test was shaken by hand 10 seconds to permit thorough mixing of the ingredients. The tests were read immediately and then placed in the shaking machine for 15 seconds

$\pm$	+	+	+	1
++ ++ +	++ ++ +	++ ++ +	++ ++	-
$\pm$	+	+	+	1
++ ++ +	++ ++ +	++ ++	++ ++	-
$\pm$	+	$\pm$	$\pm$	1
++ ++	++	+	+	-
$\pm$	+	$\pm$	$\pm$	1
++ ++	++	$\pm$	+	-
}				
1	2	5	1	15

and the results read again. The tests were then shaken for periods of 30, 60, 60, 60 and 120 seconds, respectively, the results being recorded after each shaking period. Table 25 illustrates the findings with 4 sera.

Practically all weakly positive sera showed optimum precipitation after about 2 minutes' shaking. As was true in our previous experiment, a 3 or 4 minute shaking period gave slightly stronger reactions in isolated cases.

Experiments on the effect of prolonged shaking indicated that, when the period was extended to 8 minutes, some precipitation reactions became slightly weaker.

In the choice of a shaking period for the routine test, the aim was—as in the case of the other steps of the procedure—not to render the test of the highest sensitiveness but rather of a conservative degree of sensitiveness combined with practicability. Based on this consideration, a 2 minute shaking period was chosen.

PART III

SPECIAL STUDIES ON AUTHOR'S TEST

CHAPTER X

COMPONENT OF ANTIGEN DILUTION RESPONSIBLE FOR SPECIFIC PRECIPITATION

The antigen dilution employed in the author's test consists of approximately equal parts of an especially prepared cholesterinized antigen and salt solution. The final mixture, as already indicated, contains a heavy white precipitate. It seemed worth while to separate this precipitate by centrifugation and study its behavior, as well as that of the supernatant fluid, with various sera.

EXPERIMENTAL

The following antigen dilutions and controls were prepared.

Antigen dilution 1. Standard antigen dilution was prepared by mixing 1 cc. antigen with 1 cc. salt solution (based on titration) in the usual manner.

Special antigen dilution 2. Precipitate of standard antigen dilution was suspended in a solution consisting of equal parts of 95 per cent alcohol and salt solution. This dilution was prepared as follows. One cubic centimeter antigen was mixed with 1 cc. of salt solution in the usual manner and the mixture centrifuged at high speed for 15 minutes. The supernatant fluid was pipetted off and the amount replaced with a solution prepared by mixing equal amounts of 95 per cent alcohol and salt solution. The alcohol-saline solution was employed to simulate the medium in which the precipitate of the standard antigen dilution is suspended which is essentially a solution of equal amounts of 95 per cent alcohol and saline.

TABLE 26
Precipitation reactions with sera employing component parts of standard antigen dilution

	ANTIGEN 1	ANTIGEN 2	ANTIGEN 3	ANTIGEN 4	ANTIGEN 5	ANTIGEN 6
Type of antigen.....	Standard anti- gen dilution (control)	Precipitate of standard antigen dilution sus- pended in alcohol- saline solution	Precipitate of standard antigen dilution sus- pended in one-half normal saline	Supernatant fluid of standard an- tigen dilution	Alcohol-saline solution (control)	One-half nor- mal saline (control)
Antigen, cc.....	0.025	0.025	0.025	0.025	0.025	0.025
Serum, cc.....	0.15	0.15	0.15	0.15	0.15	0.15
Specimen number	Results					
1-10	++++	++++	++++	—	—	—
11	++++	++++	+++	—	—	—
12-13	++++	++++	+++	—	—	—
14	++++	++++	++	—	—	—
15	++++	++++	+	—	—	—
16	++++	++++	—	—	—	—
17	+++	+++	+	—	—	—
18-19	++	++	+	—	—	—
20-21	+	+	—	—	—	—
Negative sera, 22-28	—	—	—	—	—	—
Salt solution, 29-30	—	—	—	—	—	—

Special antigen dilution 3. Precipitate of standard antigen dilution was suspended in a solution consisting of equal parts of distilled water and salt solution. This dilution was prepared by centrifuging 2 cc. of standard antigen dilution at high speed as above, pipetting off the supernatant fluid and replacing it with an equal amount of a solution consisting of 1 part of distilled water to 1 part of salt solution.

Special antigen dilution 4. Supernatant fluid of standard antigen dilution was pipetted off after throwing down the precipitate by heavy centrifugation.

Alcohol-saline solution (control) 5. This was prepared by mixing equal amounts of 95 per cent alcohol and salt solution.

Distilled water-saline solution (control) 6. A solution consisting of equal amounts of distilled water and salt solution.

Antigen dilutions and controls (1 to 6) were tested with a group of positive and negative sera. The tests were carried out by adding 0.15 cc. of the same serum to 0.025 cc. of each of the 6 mixtures. The tubes were shaken for 2 minutes in the usual manner, 0.5 cc. salt solution added to each, and all were observed for precipitates. The results with 28 sera are recorded in table 26.

DISCUSSION OF RESULTS

It is evident from table 26 that the special antigen dilution 2, prepared by suspending the precipitate from the standard antigen dilution in a solution containing equal amounts of 95 per cent alcohol and saline, gave results practically identical with those given by the standard antigen dilution. This indicates that the precipitate, not the supernatant fluid, of the standard antigen dilution is responsible for the specific reactions with sera. The results further indicate that the physical state of the pre-

cipitate when suspended in the approximately 50 per cent alcohol solution is similar to that of the precipitate in the standard antigen dilution.

This falls in line with our earlier studies, already emphasized, that it is not the quantity of antigenic lipoids alone which is responsible for sensitive precipitation reactions with syphilitic sera, but the physical state of these lipoids as well. What is particularly required is that the antigen-saline dilution be in a highly unstable condition as is illustrated in the standard antigen dilution which contains a precipitate readily soluble in serum. When the antigen precipitate is suspended in approximately 50 per cent alcohol, the final mixture apparently meets the above requirements with the result that the reactions with syphilitic sera approach in sensitiveness the reactions given by the standard antigen dilution.

The special antigen dilution 3 prepared by suspending the standard antigen-dilution precipitate in a solution containing equal amounts of distilled water and saline was found to be less sensitive than the standard antigen dilution. The physical condition of this antigen dilution differed from that of the standard antigen dilution in that the precipitate, instead of being heavy and unmistakable, was in a finely divided state, the mixture appearing turbid but with no definite particles visible. The fact that the antigen precipitate was in a semi-dissolved state undoubtedly helps explain the less sensitive reactions with sera.

It should be stated in this connection that the antigen-dilution precipitates discussed early in these studies (table 5) which were readily soluble in normal as well as half normal saline is not to be confused with the standard antigen-dilution precipitate employed in these experiments. The early antigen was prepared by nearly complete removal of the ether extractives and by final cholesterinization with but 0.4 per cent of cholesterin. The present standard

antigen, however, is prepared by removing only a minimum amount of the ether extractives which conforms with the use of the antigen in the test and, furthermore, 0.6 per cent cholesterin is employed. These changes in the antigen preparation necessitate the use of a larger amount of salt solution to entirely dissolve the antigen precipitate than that used in these experiments.

The negative results with the various sera given by the supernatant fluid obtained after removing the precipitate from the standard antigen dilution would indicate that this fluid is practically free from antigenic material. The results with the alcohol-saline control as well as with the half normal saline control were negative, as might have been expected.

Of interest also is the fact that the special antigen dilutions 2 and 3 have only experimental value and cannot be used in regular tests with serum. The reason for this is that, when the precipitates from the standard antigen dilution are suspended in approximately 50 per cent alcohol or half normal saline, cholesterin crystals soon begin to separate out. Cholesterin, as is well known, is comparatively insoluble in weak alcoholic solutions and entirely insoluble in watery solutions. When transferring, therefore, the antigen precipitate into the new mediums, the weakly combined cholesterin is apparently incapable of remaining in solution. The result is that, when mixed with sera, cholesterin crystals precipitate out and interfere with the reading of the specific precipitates.

CHAPTER XI

EFFECT OF INCUBATION ON THE TEST

Practically all biological reactions are affected by incubation, being usually either enhanced or reversed. The fact that practically all reactions with the author's test are completed within several minutes after mixing serum with antigen dilution, renders the study of the effect of incubation of especial interest. It was indeed believed that studies on incubation might throw some light on the degree of specificity of the test. Thus, if incubation tends to produce precipitation in all sera—including non-syphilitic—it would seem likely that we are dealing with a test possessing but relative specificity; the difference between syphilitic and non-syphilitic sera being merely the degree of rapidity with which precipitation is brought about. If, on the other hand, non-syphilitic sera remain free from precipitates even after prolonged incubation, it would be reasonable to believe that we are dealing with a test of high specificity and that the negative reactions indicate a lack of syphilitic reacting substances.

EXPERIMENTAL

The effect of incubation was studied with a total of 882 sera. The tests were carried out in the usual manner, employing 0.15 cc. of serum and 0.05, 0.025 and 0.0125 cc. of antigen dilution, respectively. After shaking the tests 2 minutes, readings were made immediately and after 1, 4 and 24 hours' incubation at 37°C. In one group of sera (542), 0.5 cc. salt solution was not added to each tube before the incubation periods. In the remaining group of sera (340), 0.5 cc. salt solution was added to each tube

after the two minute shaking period and the effect of incubation in the presence of salt solution studied.

In order to overcome the element of evaporation, particularly in the series which was incubated without the ad-

TABLE 27
Effect of incubation on test
Before addition of salt solution to tubes

NUMBER OF SERA	INCUBATION PERIOD			
	None	1 hour	4 hours	24 hours
	Results—Average of findings in three tubes			
385	—	—	—	—
133	++++	++++	++++	++++
3	—	—	—	+
2	—	—	+	+
1	—	+	+++	+++
1	—	+	++++	++++
1	+	+	++	++
1	+	+	+	+++
1	+	++	++	++
2	+	+++	++++	++++
1	+	+++	++++	++
1	+	++++	++	++
1	++	++	++++	++++
1	++	+++	+++	++++
1	++	++++	++++	++++
1	++	+	+	—
2	++	++	—	—
1	++	+++	+	—
1	+++	+	—	—
1	+++	+++	+++	—
1	+++	+++	++	+
Total...542				

dition of salt solution, the racks were kept in covered copper containers in which were placed wet sponges during the incubation periods. It was early observed that when incubating the small amounts of serum and antigen dilution used in the test, without due protection against evaporation, the loss of fluid due to evaporation was frequently

sufficient to throw down the serum proteins, giving the impression of specific precipitates. These proteins, it is true, would, in most cases, readily dissolve again on addition of salt solution, thus distinguishing them from specific precipitates. In isolated cases, however, the serum pre-

TABLE 28
Effect of various periods of incubation on test
After addition of salt solution to tubes

NUMBER OF SERA	INCUBATION PERIOD			
	None	1 hour	4 hours	24 hours
	Results—Average of findings in three tubes			
257	—	—	—	—
61	++++	++++	++++	++++
2	+	—	—	—
1	+	+	—	—
1	++	—	—	—
1	++	+	—	—
1	++	++	+	+
1	+++	—	—	—
1	+++	+	—	—
1	+++	++	—	—
1	+++	++	++	++
2	+++	+++	+++	+
1	++++	++	+	—
1	++++	+++	++	+
1	++++	++++	++	++
1	++++	+++	+++	—
3	++++	++++	++	++
1	++++	++++	++++	++
2	++++	++++	+++	+++
Total...340				

cipitates would not redissolve in salt solution. No tendency for evaporation of the serum-antigen mixtures was observed when employing the covered containers.

Table 27 gives the results obtained with 542 both positive and negative sera incubated without the addition of 0.5 cc. salt solution to each tube. Table 28 gives the results

of 340 sera incubated after the addition of 0.5 cc. salt solution to each tube. The average of the results of the 3 tube tests is recorded in each case, this average representing the final result in the regular test.

DISCUSSION OF RESULTS

Of 542 positive and negative sera incubated without the addition of 0.5 cc. salt solution, 518 or 95.6 per cent showed no change after 24 hours' incubation. Of the remaining 24 or 4.4 per cent, 17 sera showed either slight or comparatively marked strengthening in the reaction due to incubation, particularly after the 24 hours. Seven, on the other hand, gave weaker reactions after incubation.

Of 340 sera incubated after the addition of 0.5 cc. salt solution, 318 or 93.5 per cent showed no change. The remaining 22 sera or 6.5 per cent showed a tendency toward weakening of the reactions. This finding might perhaps have been expected when taking into consideration the effect of dilution on precipitation with syphilitic sera which was fully discussed in Chapter IV.

Of particular interest, in our opinion, are the incubation results obtained under conditions of concentration, without the addition of salt solution. Eliminating the 5 negative sera which became only one plus after incubation, only 2 in a total of 385 negative sera became definitely positive after incubation. One of these 2 sera came from a treated case of syphilis, the source of the other we have not been able to locate. Whether or not the precipitation reaction with this serum following incubation was specific does not affect the evident indication that incubation, even when prolonged to 24 hours, shows no tendency for the formation of false precipitates in the author's test. This, as already indicated, speaks for the inherent specificity of the test. If the test were only relatively specific and the gradation between positive and negative sera based on a fine rather

than more definite demarcation, it would be reasonable to expect that many, if not all, negative sera would give precipitation reactions after prolonged incubation.

The fact that some reactions become stronger after incubation and some weaker is also of interest and deserves further study. There may indeed exist some relation between the behavior of the serum after incubation and the clinical condition of the patient. Lack of clinical material has not enabled us to study these fluctuations in the reactions from the clinical viewpoint. This apparently is clear—the effect of incubation at 37°C. on the test is comparatively little.

CHAPTER XII

EFFECT OF ACID AND ALKALI ON TEST

It was observed in connection with our early precipitation studies that the addition of comparatively large quantities of acid to negative sera caused them to give precipitation reactions with antigen dilution, while alkali added to positive sera rendered them negative. This observation seemed of practical importance since improperly cleaned glassware may contain traces of acid or alkali. Experiments were thereupon carried out to determine the quantities of acid and of alkali which, when added to the different ingredients of the test, would produce non-specific results.

EXPERIMENTAL

Experiment 1. Acid added to salt solution. To a series of 1 cc. quantities of salt solution were added varying amounts of acid, making final concentrations ranging from 0.1 to 12.5 per cent normal HCl. The range between 0.1 to 1 per cent acid was increased by gradations of 0.1 per cent and between 1 to 12.5 per cent acid by gradations of 0.5 per cent, making a total of 33 solutions. When the salt solutions containing from 0.1 to 0.5 per cent normal acid were mixed in 0.15 cc. amounts with 0.025 cc. quantities of standard antigen dilution, no resulting precipitates were observed, while in the higher concentrations, precipitates were visible in every case. After shaking for 2 minutes, the precipitates resulting from mixing the salt solutions containing from 0.5 to 1.5 per cent acid with antigen dilution became considerably weaker, while those of the higher concentrations of acid were found to be unaffected by this shaking period.

TABLE 29
Effect of adding acid to negative sera

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6	TUBE 7	TUBE 8	TUBE 9	TUBE 10	TUBE 11
HCl N/1 added to serum, per cent.....	0	2	3	4	5	6	7	8	9	10	12.5
Antigen dilution, cc.....	0.025 } in each tube 0.15 }										
Serum, cc.....											
Serum number	Results										
1	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+
11*	+	+	+	+	+	+	+	+	+	+	+

* Positive serum.

Experiment 2. Acid added to serum. Preliminary experiments indicated that negative serum containing as much as 2 or 3 per cent normal HCl does not produce precipitates after mixing with standard antigen dilution. A number of negative sera were thereupon acidified as follows: Each serum was divided into ten parts and made up to contain 2, 3, 4, 5, 6, 7, 8, 9, 10 and 12.5 per cent concentration of normal HCl, respectively. Regular precipitation tests were then carried out employing 0.15 cc. amounts of each of the acidified sera with 0.025 cc. standard antigen dilution.

As indicated in table 29, 2 and 3 per cent of normal acid had no effect on the sera. At 4 per cent, a few sera showed weak precipitates after mixing with antigen dilution. At 5, 6 and 7 per cent acid, practically all sera showed precipitates. Beyond this, a zone phenomenon was observed, many sera showing weaker precipitates at 8, 9 and 10 per cent with stronger reactions at 12.5 per cent.

Experiment 3. Acid added to antigen. A series of 1 cc. quantities of antigen were made up to contain 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 10 and 12.5 per cent concentration of normal HCl, respectively. These acidified antigens were then diluted with salt solution in the usual manner. The antigen dilutions, in 0.025 cc. quantities, were mixed with 0.15 amounts of normal saline to test the solubility of the antigen dilution precipitates. It was observed that the precipitates in the antigen dilutions prepared from the acidified antigens ranging from 0.5 to 3 per cent normal HCl were soluble in salt solution, while those in the dilutions prepared from the antigens of higher acid concentration were insoluble. When tested with negative sera, the antigen dilutions containing precipitates which were soluble in salt solution showed no tendency for acid precipitation. The antigen dilution precipitates which were insoluble in salt solution were also insoluble in serum. It is of

interest that in a few cases 2 minutes' shaking tended to dissolve the precipitates.

In a similar experiment, acidified salt solution (instead of acidified antigen) was used in the preparation of a series of antigen dilutions. As in the previous experiment, the concentration of normal acid ranged from 0.5 to 12.5 per cent. The results were practically identical to those obtained when acidifying the antigen.

Experiment 4. Alkali added to salt solution. To a series of 1 cc. quantities of normal saline were added varying amounts of alkali. The final concentrations of normal NaOH in the salt solutions ranged from 0.5 to 12.5 per cent as in the case of the acid experiments. These solutions, in 0.15 cc. quantities, were added, in series, to 0.025 cc. amounts of standard antigen dilution. The antigen dilution precipitates immediately dissolved. It was then desired to find the effect of increasing the concentration of alkali in the salt solution. It was observed that alkali concentrations ranging from 12.5 per cent normal NaOH to 40 per cent continued to dissolve the precipitates of the standard antigen dilution. In higher concentrations of alkali—prepared by adding various amounts of 4 normal NaOH to a series of 1 cc. quantities of salt solution—up to 100 per cent normal NaOH, the precipitates of the antigen dilution were found to be insoluble. Of interest is the fact that 2 minutes' shaking dissolved the precipitates resulting from mixing antigen dilution with the salt solutions containing from 40 to 60 per cent normal NaOH. Beyond 60 per cent alkali concentration the precipitates remained stable, even after the shaking period.

Experiment 5. Alkali added to serum. Preliminary experiments indicated that 1 per cent normal NaOH could be added to positive serum with no affect on the results of the test. To a series of positive sera was added alkali to give final concentrations of 1, 1.25, 1.66, 2.5 and 5 per

cent normal NaOH. These sera, in 0.15 cc. amounts were added to 0.025 cc. quantities standard antigen dilution. As indicated in table 30, 2.5 per cent alkali was sufficient to render a large number of positive sera negative while 5 per cent rendered negative all the sera tested.

Experiment 6. Alkali added to antigen. Alkali was added to a series of 1 cc. quantities of antigen to make final concentration in terms of normal NaOH ranging from 1 to 50 per cent. Antigen dilutions were prepared

TABLE 30
Effect of adding alkali to positive sera

	TUBE 1	TUBE 2	TUBE 3	TUBE 4	TUBE 5	TUBE 6
NaOH N/1 in serum, per cent.....}	0	1.0	1.25	1.66	2.5	5.0
Antigen dilution, cc.....	0.025 } 0.15 } in each tube					
Serum, cc.....						
Serum number	Results					
1	+++++	+++++	+++++	++	—	—
2	+++	++	±	—	—	—
3	+++++	+++++	+++++	+++++	±	—
4	++	++	±	—	—	—
5	+++++	+++++	+++++	+++++	+++++	—
6	+++	+++	+++	++	—	—
7	+++++	+++++	+++++	+++++	—	—
8	+++++	+++++	+++++	+++++	—	—
9	+++++	+++++	+++++	+++++	—	—
10	+++++	+++++	+++++	+++++	+++++	—
11*	—	—	—	—	—	—

* Negative serum.

in the usual manner from this series and each tested in 0.025 cc. quantities with six different positive sera and a salt solution and negative serum control, all in 0.15 cc. quantities. It was observed that the precipitates in the antigen dilutions prepared from the antigens containing concentrations of normal NaOH ranging from 1 to 12.5 per cent were soluble in the salt solution and negative serum control and the reactions with all the positive sera

remained unchanged. Beyond this alkali concentration, the antigen dilution precipitates were insoluble in salt solution and also in negative serum, and therefore the reactions with positive sera could not be studied.

In an experiment covering the same concentrations of alkali in the salt solution (instead of the antigen), used for preparing antigen dilution, similar results were obtained.

DISCUSSION OF RESULTS

The results of these studies, although preliminary in character, indicate that inorganic acid and alkali, in sufficient concentration, have a marked effect on the author's test.

Negative serum containing 3 per cent normal HCl gives precipitates when used with standard antigen dilution. Salt solution, on the other hand, containing as little as 0.5 per cent normal acid substituted for the serum shows precipitates with antigen dilution. The comparatively large amount of acid in serum necessary to bring about precipitation with antigen dilution is undoubtedly due to the buffer action of the serum. Of special interest is the observation that many sera show zone phenomena in the higher acid concentrations, while acidified salt solution shows no tendency towards zone phenomena.

Normal NaOH appears to exert no influence on the reactions of positive serum until the alkali concentration reaches 2.5 per cent. At this concentration, some sera give negative reactions with antigen dilution. When the concentration of alkali is increased to 5 per cent, all the positive sera tested gave negative results. No zone phenomena were observed when the alkali concentration was increased beyond 5 per cent and up to 40 per cent. At this high alkali concentration the antigen dilution precipitate apparently lost its property of dissolving in salt solution or serum.

CHAPTER XIII

USE OF DIFFERENT ANTIGENS IN TEST

It was observed in the course of our studies that, when applying the principles of our precipitation procedure to differently prepared antigens, precipitation results approaching those given by the standard antigen were obtained. It was believed important at this stage of our studies to verify this observation by quantitative experiments.

EXPERIMENTAL

The following 7 antigens were prepared for comparative study:

Antigen 1. This antigen was essentially standard antigen to which was added acetone insoluble lipoids obtained from the ether extract and from a secondary alcoholic extract of the beef heart.

Preparation. Twenty-five grams powdered beef heart were placed in a 250 cc. Erlenmeyer flask, and the ether extraction carried out according to the standard procedure with 100, 75, 75, and 75 cc. quantities of ether, respectively. The final ether extract was saved. The beef heart was then extracted with 95 per cent alcohol, 5 cc. per gram, for 3 days at room temperature and the extract filtered off.

A second alcoholic extract was prepared by extracting the beef heart residue with 95 per cent alcohol, 3 cc. of alcohol per gram of material. This extraction was carried out for 3 days at room temperature followed by 1 day in the incubator at 37°C., at the end of which period the extract was filtered off.

The ether extract and the second alcoholic extract were evaporated to dryness by revolving fans. Both residues

were taken up in about 15 cc. of ether and the insoluble elements filtered off. To the clear ether filtrate were added 250 cc. acetone and the mixture was permitted to stand over night in the icebox. After decanting the supernatant acetone as completely as possible, the residue was redissolved in 10 cc. ether and the solution added slowly to the first alcoholic extract. The final product was permitted to stand for a day and filtered. To a portion of this antigen were then added 6 mgm. of cholesterin per cubic centimeter.

Antigen 2. Kolmer antigen (30), which consists of a secondary alcoholic extract of powdered beef heart to which is added acetone insoluble lipoids obtained from the ether extract and from the first alcoholic extract, was prepared. Kolmer's latest technic was employed. The final product contained 0.2 per cent cholesterin.

Antigen 3. To Kolmer's antigen was added 0.4 per cent cholesterin to give a final concentration of 0.6 per cent cholesterin.

Antigen 4. A Noguchi or acetone insoluble antigen was prepared from powdered beef heart.

Preparation. Twenty-five grams of powdered beef heart were placed in a 250 cc. Erlenmeyer flask and 125 cc. 95 per cent alcohol added. This was extracted for 3 days at room temperature and 1 day in the incubator at 37°C. After filtration, the alcoholic extract which totalled 80 cc. was evaporated to dryness. The dry residue was dissolved in 15 cc. ether and the insoluble particles removed by filtration. To the clear ether extract were added 250 cc. acetone, and the mixture permitted to stand over night in the icebox. The supernatant acetone was decanted as completely as possible and the residue dissolved in 80 cc. absolute alcohol. To a portion of this antigen were added 6 mgm. cholesterin per cubic centimeter.

Antigen 5. Standard antigen used in the author's test was employed.

Antigen 6. A secondary alcoholic extract of powdered beef heart was prepared.

Preparation. After preparing standard antigen from 25 grams of powdered beef heart, the heart muscle residue was re-extracted with 95 per cent alcohol in the proportion of 3 cc. per gram of powder. The extraction was carried out for 3 days at room temperature and 1 day in the incubator at 37°C. After filtration, 6 mgm. cholesterin were added per cubic centimeter of extract.

Antigen 7. An acetone insoluble extract of ether extractives of beef heart was prepared.

Preparation. The ether extract from the preparation of 25 grams of powdered muscle was evaporated to about 10 cc. Two hundred cubic centimeter acetone were added, and the mixture permitted to stand over night in the icebox. The supernatant acetone was poured off and the residue shaken up in 25 cc. absolute alcohol. After removal of the insoluble elements by filtration, the extract was cholesterinized by adding 6 mgm. cholesterin per cubic centimeter.

Antigens 1 to 7 were now titrated with salt solution, employing the standard titration procedure described in Chapter XVII. The aim was to determine the minimum amount of salt solution to mix with antigen which would produce a precipitate soluble in salt solution. The titres of these antigens were found to be as follows:

Antigen 1.	1 cc. antigen + 1	cc. salt solution
Antigen 2.	1 cc. antigen + 0.9	cc. salt solution
Antigen 3.	1 cc. antigen + 1.1	cc. salt solution
Antigen 4.	1 cc. antigen + 1	cc. salt solution
Antigen 5.	1 cc. antigen + 1	cc. salt solution

Antigens 6 and 7 were found to be unfit for use due to the presence of cholesterin crystals after addition of salt solution.

It was observed that some crystallization of cholesterin took place in all antigens except no. 5, after diluting them with salt solution and permitting them to stand for 10 or 15 minutes at room temperature. In the case of antigen 5, no cholesterin crystals were observed 45 minutes after diluting it with salt solution.

TABLE 31
Comparative sensitiveness of different antigens in author's test

	ANTIGEN 1	ANTIGEN 2	ANTIGEN 3	ANTIGEN 4	ANTIGEN 5
Type of antigen..	Alcoholic, with additional acetone insoluble lipoids	Kolmer	Kolmer, with additional cholesterin	Acetone insoluble	Kahn
Number of sera	Results				
10	++++	++++	++++	++++	++++
2	++++	+++	++++	++++	++++
3	++++	++	++++	++++	++++
2	++++	+	++++	++++	++++
2	+++	±	++++	++++	++++
1	++	±	+++	+++	+++
1	+	±	+++	+++	+++
1	+	—	++	++	++
2	—	—	+++	++	+++
1	—	—	++	±	+++
1	—	—	+	+	++
1	—	—	++	±	+
1	—	—	+	±	+
4	—	—	+	—	+
8	—	—	—	—	—

This tendency for the crystallization of cholesterin after diluting antigens 1 to 4 with salt solution could have been reduced by the employment of somewhat larger amounts of salt solution for antigen dilution. Thus, an antigen diluted with salt solution in the proportion of 1 + 1 cc. may show the presence of cholesterin crystals 15 minutes after standing, whereas the same antigen diluted with a somewhat larger amount of salt solution may not show these crystals until after 30 minutes' standing. Needless

to say, the presence of these crystals interferes with correct reading of results when testing with serum.

Since, however, increasing the amount of salt solution for diluting antigen tends to decrease the sensitiveness of the antigen with sera, it was thought best to employ antigens 1 to 4 in the titres indicated and to employ them in the serum tests within 10 minutes after their dilution with salt solution—an interval in which no cholesterin crystals were observed.

The following procedure was employed for testing the comparative sensitiveness of antigens 1 to 5 with sera. The antigens were diluted with salt solution according to the titres indicated. Each antigen dilution was employed in a 0.025 cc. amount. The sera, in 0.15 cc. quantities, were then added to each of the 5 antigen dilution amounts. The tests were shaken 2 minutes; 0.5 cc. salt solution added to each tube and the results read.

As indicated in table 31, the more strongly positive sera showed complete precipitation with all the 5 antigens used. In the case of the weaker sera, the Kolmer antigen containing 0.6 per cent cholesterin and the acetone insoluble antigen, also containing 0.6 per cent cholesterin, were practically as sensitive as the standard antigen.

DISCUSSION OF RESULTS

The results of this experiment indicate that the principles governing precipitation with syphilitic sera presented in this volume are not limited in their application to a single antigen but apply to other antigens as well.

Our early studies have shown that certain types of antigens can not be employed in the author's test. Extracts prepared from wet heart muscle are unsatisfactory, due to insufficient concentration of specific lipoids; crude alcoholic extracts of dried muscle can not be used because they produce a stable precipitate when diluted with salt solution. These

antigens, it is true, produce precipitates with syphilitic sera under certain conditions, but the results do not approach in sensitiveness the results with standard antigen. Antigens which have undergone some degree of purification, however, either by the removal of acetone soluble or ether soluble lipoids, may give results comparable in sensitiveness with those given by standard antigen.

There are other factors, however, aside from sensitiveness which must be taken into consideration. The behavior of an antigen after dilution with salt solution may render an antigen unsuited for the test. Antigens 3 and 4, for example, employed in this experiment, as already indicated, show cholesterin crystals about 10 minutes after dilution with salt solution and are thus impracticable in routine work. The method of preparation of an antigen must also be considered. It is of particular importance that the method assure a uniform product. While antigens 3 and 4, just mentioned, give reactions similar to the standard antigen, it is evident that their method of preparation would not be likely to lead to the same degree of uniformity as the standard antigen.

It is of interest that antigens 6 and 7 consisting of a secondary alcoholic extract of beef heart and an acetone insoluble extract of ether lipoids, respectively, could not be diluted with salt solution without immediately throwing down cholesterin crystals. Our explanation for this is the probable necessity for the presence of a comparatively large amount of lipoids to keep the lipid cholesterin in solution after diluting the antigen with salt solution. Antigens 6 and 7 contain apparently an insufficient amount of lipoids to hold the cholesterin in solution after the addition of salt solution.

CHAPTER XIV

AN ESPECIALLY SENSITIVE ANTIGEN

In the discussion of the factors which affect the sensitiveness of the author's standard antigen (Chapter VII,) it was shown that variations in the method of the preliminary ether extraction had a marked effect on the sensitiveness of the antigen, while variations in the alcohol extraction method had little effect. Thus, an antigen in which the ether extraction was excessive was far less sensitive than one in which it was limited. It seemed of importance to determine by quantitative experiments to what extent the sensitiveness of an antigen could be increased by limiting the ether extraction.

It will be recalled that our aim in the preparation of a standard antigen was not to render it of the greatest sensitiveness but rather of a degree of sensitiveness conforming with safety in a diagnostic method. It seemed possible that an antigen possessing a somewhat greater degree of sensitiveness than the standard antigen—if it could be prepared—might form the basis of a procedure for the presumptive diagnosis of syphilis. Such a procedure might prove a valuable check on the routine test, particularly in the case of the weakly positive or doubtful reactions. It might also prove valuable in cases undergoing antisyphilitic treatment and in special cases of syphilis in which the routine test is negative.

EXPERIMENTAL

Preliminary experiments indicated that the amount of ether employed in the beef heart extraction could be somewhat reduced; further, that the 10 minute extraction

periods employed in the preparation of standard antigen could be changed to 5 minute periods. Four antigens were thereupon prepared which varied from the standard antigen only in the method of the ether extraction.

Antigen 1. The ether extraction was carried out with 75, 50, 50 and 50 cc. quantities of ether, respectively, each ether "washing" being limited to 5 minutes.

Antigen 2. The ether extraction was carried out with 50, 50, 40 and 40 cc. of ether, respectively, employing 5 minute washing periods.

Antigen 3. The ether extraction was carried out with 50, 50, 35 and 25 cc. ether quantities, respectively, with washings at 5 minute periods.

Antigen 4. The ether extraction was carried out with 50, 25, 25 and 25 cc. ether quantities, respectively, with washings at 5 minute periods.

These four antigens were titrated with salt solution in the usual manner. It was observed that when mixing antigens 3 and 4 with varying amounts of salt solution a precipitate was formed which was insoluble on further addition of salt solution. These two antigens, therefore, could not be used. In the case of antigens 1 and 2, it was observed that, when diluted with salt solution, both produced precipitates which were soluble on further addition of salt solution. Either of them, therefore, could be used for comparative study with the standard antigen. Antigen 2 was chosen because it contained the relatively larger amount of ether extractives.

When titrating antigen 2, it was found that 1 cc. antigen + 1.3 cc. salt solution resulted in an antigen dilution which conformed with the requirements of the titration. The titre of the standard antigen used was 1 cc. + 1 cc. salt solution. The antigen dilutions prepared from these 2 antigens were tested, in a preliminary experiment, with 200 syphilitic and non-syphilitic sera. It was found that

antigen 2 gave five more positive reactions than the standard antigen. Four of these reactions were obtained with specimens from cases undergoing antisyphilitic treatment. The fifth was obtained with a specimen from a case of unknown history.

To obtain a more quantitative difference between the sensitiveness of the two antigens, the following experiment was carried out.

TABLE 32
Comparative findings with standard and special antigens

	ANTIGEN					
	Standard			Special (antigen 2)		
	1:10	1:20	1:30	1:10	1:20	1:30
Serum dilutions.....						
Serum:antigen dilution....	6:1 in each tube					
Number of sera	Results					
1	+++++	+++++	—	+++++	+++++	—
1	+++++	++	+	+++++	+++++	+
1	+++++	++	+	+++++	+++	++
1	+++++	++	—	+++++	+++	+
1	+++++	++	—	+++++	++	+
1	+++++	±	—	+++++	±	—
2	+++++	—	—	+++++	±	—
1	+++++	—	—	+++++	—	—
1	+++	+	—	+++++	++	—
1	+++	—	—	+++++	+	—
1	+	—	—	++	—	—
10	—	—	—	—	—	—

Experiment. In order to reduce the number of reacting substances in a definite range, a number of syphilitic sera were diluted with salt solution in the proportions of 1:10, 1:20 and 1:30, respectively. The 3 dilutions of each serum were subsequently tested with both antigens, employing in each case 0.025 cc. of antigen dilution and 0.15 cc. of diluted serum. The tests were read after the usual two minute shaking period. Table 32 gives the results of 12 positive and 10 negative sera tested.

It is evident from this table that antigen 2 gives stronger reactions than the standard antigen. Further studies corroborated these findings and indicated that antigen 2 was capable of detecting minute amounts of reacting substance in syphilitic sera. It was believed that an antigen of such sensitiveness would give occasional false reactions with sera, since a biologic test which is sufficiently sensitive to detect traces of reacting substance might be expected to give non-specific reactions. The infrequency of such reactions with antigen 2 was indicated by experiments already discussed and by further comparative examinations with the routine tests. It seemed therefore that this antigen might form the basis of a highly sensitive presumptive procedure.

In developing a procedure of this kind, the use of a single serum-antigen proportion suggested itself. It did not seem necessary to render a presumptive test quantitative by employing more than one serum-antigen proportion. The selection of this proportion was based on knowledge gained from our studies on the quantitative relations between serum and antigen (Chapter VI). It was shown in these studies that an excessive amount of antigen rendered a positive reaction negative. A serum containing but a small number of reacting substances gave a complete precipitation reaction only with a small amount of antigen. It is evident from this that the 3:1, 6:1 and 12:1 proportions of serum to antigen dilution employed in the routine test were not chosen with a view of producing reactions of the highest sensitiveness, since some sera giving negative reactions in the 12:1 proportion would give positive reactions in an 18:1 or 24:1 proportion. The use of such proportions in the routine test, however, was impracticable for, aside from the possibility of non-specific reactions, the comparatively small quantity of antigen present often rendered the precipitate formed in positive sera so fine as to be hardly visible.

It was observed that antigen 2, containing a larger amount of ether-soluble lipoids, produced somewhat heavier precipitates with sera than the standard antigen. This suggested the possibility, in the employment of this antigen in a presumptive procedure, of using a slightly higher serum-antigen proportion than is used in the routine test. It was believed that a 15:1 proportion might prove practicable.

From the results of the experiments with shaking, discussed in Chapter IX, it seemed that the sensitiveness of

TABLE 33

Comparative sensitiveness of standard and special antigens when employing technic of presumptive procedure

	ANTIGEN	
	Special (antigen 2)	Standard
Serum:antigen dilution	15:1 in each tube	
Number of sera	Results	
52	++++	++++
2	++++	+++
1	++++	++
3	+++	++
2	+++	+
2	+++	±
4	++	+
4	+	—
10	—	—

the presumptive procedure might be further increased by extending the shaking period employed in the routine test. It was shown that a three minute period gave slightly more sensitive reactions than a two minute period. Since a high degree of sensitiveness was to be the outstanding feature of the presumptive procedure, it seemed best to adopt a 3 minute shaking period.

The following experiment was carried out with a view to determining the comparative sensitiveness between antigen 2 and the standard antigen, employing a 15:1 pro-

portion of serum to antigen dilution and a 3 minute shaking period.

Experiment. Antigen dilutions were prepared from antigen 2 and the standard antigen. These dilutions were pipetted in 0.01 cc. amounts in a series of tubes. Syphilitic and non-syphilitic sera were then pipetted in 0.15 cc. amounts. The tests were shaken for 3 minutes, 0.5 cc. salt solution added to each tube and the results read. It was found that antigen 2 gave more sensitive reactions than the standard antigen. It was further found that the sera which gave the same reactions with both antigens showed somewhat heavier precipitates with antigen 2 than with the standard antigen. Table 33 gives the results with 80 sera tested.

DISCUSSION OF RESULTS

A consideration of the factors governing sensitiveness of antigen led to preparation of an antigen possessing greater sensitiveness than the standard. It was believed that such an antigen might form the basis of a highly sensitive presumptive procedure which would be of practical value in special cases. In attempting to utilize this antigen, experiments indicated that the use of a 15:1 serum-antigen ratio and the employment of a 3 minute shaking period were practicable in a presumptive procedure. The details of this procedure are presented in Chapter XXI.

PART IV
AUTHOR'S PRECIPITATION SYSTEM—
PROCEDURE

CHAPTER XV

GLASSWARE AND APPARATUS

Although the test permits considerable flexibility in the use of glassware and apparatus, it is well, in order to assure uniformity, to employ the standard equipment described below.

The standard tubes used for diluting the antigen are 5.5 cm. in length and 1.5 cm. in diameter. The test tubes used in carrying out the test are 7.5 cm. in length and 1 cm. in diameter. The pipettes employed are 1 cc. graduated in 0.1 or 0.01 cc. quantities and 0.2 cc. graduated in 0.001 cc. quantities (plate I). Other glassware used in the test—10 cc. pipettes, 250cc. Erlenmeyer flasks, 100 cc. cylinders, funnels of various sizes, etc.—are such as are ordinarily found in serologic laboratories. Filter paper used in antigen filtration should be of high grade, S. & S. no. 597, for example.

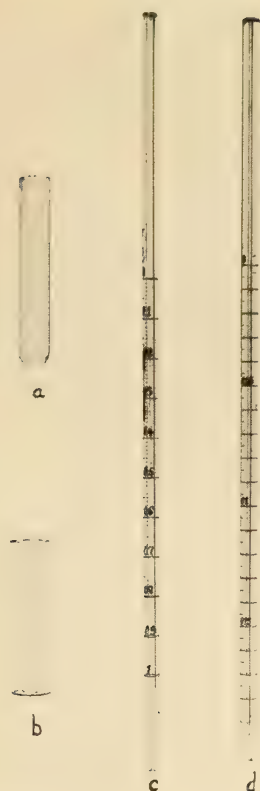
Plate II shows the standard test tube rack. The middle row of holes is offset to permit all three tubes of each test to be observed at the same time without removal from the rack. The rack, further, is so constructed as to permit thorough agitation of the serum-antigen mixtures during the shaking period.

Plate III shows a shaking machine built to hold from one to six standard racks. Although a shaking machine is not essential for a small number of tests, it is of great convenience when a large number of tests are run.

In the washing of the glassware, the aim should be thorough cleanliness combined with neutrality. In this laboratory, the process is as follows: The tubes are rinsed three times in cold tap water. After placing them for

two hours in a one per cent hydrochloric acid solution, the tubes are rinsed in tap water six times, fresh water being used each time. After rinsing twice in distilled water, the tubes are placed bottom side up in wire baskets and dried in a hot air oven. The pipettes are kept overnight in cleaning solution (sulphuric acid + potassium permanganate) and are then thoroughly rinsed and allowed to remain in an excess of running tap water for several hours. They are then rinsed twice in distilled water and dried in a hot air oven.

The importance of the neutrality of the glassware can be readily understood from the results of our studies on the effect of acid and alkali on the test (Chapter XII). A trace of cleaning solution adhering to a pipette may be responsible for incorrect results.



(Photo by L. C. Hulbert, Mich. Dept. of Health)

TEST TUBES AND PIPETTES

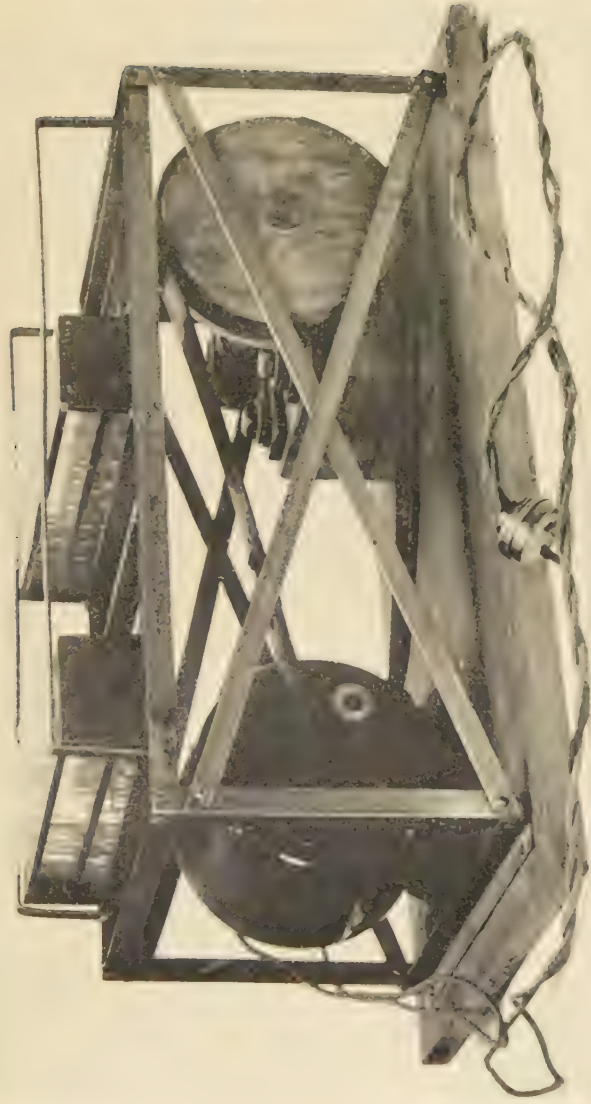
- a.* Test tube used in carrying out precipitation tests. Inner diameter about 1 cm. and length about 7.5 cm.
- b.* Test tube employed in the preparation of standard antigen dilution. Inner diameter 1.5 cm. and length about 5.5 cm.
- c.* Pipette, 1 cc. marked in 0.01 cc.
- d.* Pipette, 0.2 cc. marked in 0.001 cc.



(Photo by L. C. Hulbert, Mich. Dept. of Health)

STANDARD RACK

Made of sheet copper, 3 inches wide by 11.5 inches long by $2\frac{3}{4}$ inches high. Consists of three shelves, upper and middle ones containing 3 rows of 10 holes, each of approximately $\frac{1}{2}$ inch diameter. The center row holes offset $\frac{1}{2}$ inch. The bottom shelf serves as support.



SHAKING MACHINE

(Photograph by L. C. Hubbert, M.D., Dept. of Hygiene)

Constructed of an angle steel frame on which rides a carrier also with 6 compartments especially fitted for six standard racks. Two guard rails hold the racks in place during the shaking period. The machine is mounted on a wooden base and is driven by a quarter horse power motor with belt drive. The carrier rides on top of a supporting frame driven by an eccentric which revolves 300 r.p.m. It is capable of shaking from one to six racks. (Devised by R. L. Burd of these Laboratories.)

CHAPTER XVI

INGREDIENTS OF TEST

ANTIGEN (PREPARATION)

The antigen is an especially prepared alcoholic extract of powdered beef heart. The method of its preparation to be described is based on the antigen studies fully considered under uniformity of antigen preparation, Chapter VII. This preparation requires the extraction of definite amounts of powdered beef heart in measured amounts of ether, after which the powder is extracted in alcohol and then cholesterin added to the final extract. The unit amount of powdered beef heart used for preparing a given lot of antigen is 25 grams and this amount is extracted in a 250 cc. Erlenmeyer flask. This unit amount yields about 75 cc. of antigen. If a larger quantity is desired, as many unit quantities are employed as necessary.

Preparation of powdered beef heart. For uniformity, not less than three beef hearts should be used for a given lot. The hearts should be as fresh as possible and the drying should be carried out rapidly to avoid undue decomposition.

About 500 grams of muscle are cut from three or more beef hearts and passed four times through a meat chopper. The finely ground material is now spread out in a thin layer on a porcelain platter or glass plate and dried by placing in front of two revolving fans. When the surface becomes sufficiently dry, after about 6 hours' exposure to the fans, the material is turned over and drying continued over night. The dried material is broken up into small pieces and drying continued until the particles are crisp and easily breakable. The material may now be ground in a mortar. The final product must be in powder form.

Powdered beef heart for preparing antigen is now obtainable on the market in the form of Bacto beef heart.¹ In our experience, the market preparation gives highly uniform results, due undoubtedly to the fact that a large number of hearts are used in making a given lot. We find it advantageous therefore to employ this product in the preparation of antigen rather than a product prepared from only three beef hearts.

Ether extraction. Twenty-five grams of powdered beef heart are placed in a 250 cc. Erlenmeyer flask. One hundred cubic centimeters of ether (anesthesia) are added and the flask is shaken at frequent intervals for 10 minutes. At the end of this period the ether is filtered off. Gentle pressure is applied to the beef heart in the funnel by means of a spatula, to assure as complete removal of the ether as possible. The filtration is completed when further pressure does not cause drops of ether to pass through the funnel. The moist beef heart is now transferred to the original flask and 75 cc. fresh ether added. The flask is again shaken at frequent intervals during a 10 minute period, and the ether filtered off as in the previous case. The heart muscle is returned to the flask a third time and again covered with 75 cc. ether. The flask is shaken from time to time during a 10 minute period and filtration carried out as previously.

The moist powder is now transferred to the Erlenmeyer flask for the fourth and last ether extraction. Seventy-five cubic centimeters ether are added to the flask, shaken for 10 minutes, and final filtration of the ether carried out. The first three ether filtrations may be carried out with the same filter paper. For the last filtration, however, a fresh layer of filter paper is employed. After the ether is removed as completely as in the earlier filtrations, the moist heart muscle is spread upon a white sheet of paper or

¹ The Digestive Ferments Company, Detroit, Mich.

a clean glass plate and dried in the incubator at 37°C. for about 10 minutes or for a somewhat longer period at room temperature. When the material is dry and free from ether odor, it is ready for extraction with alcohol. The same Erlenmeyer flask in which the ether extraction was carried out may be employed for the alcohol extraction, provided the ether has been completely removed before adding the powdered muscle and alcohol.

Alcohol extraction. After completing the ether extraction as previously described, the dried powder is weighed and transferred into a 250 cc. Erlenmeyer flask. Five cubic centimeters of 95 per cent alcohol are now added per gram of powder. The flask is shaken for 10 minutes and extraction permitted for 3 days at room temperature (about 21°C.). The flask is not shaken again during this extraction period except for a 5 minute period just before filtration. The alcoholic extract after filtration is kept at room temperature in the dark as stock solution.² All corks employed in connection with the preparation and keeping of the antigen are covered with tin foil. This prevents the dissolving of alcohol-soluble elements contained in the cork. (When sending antigen through the mails, glass-sealed ampules or glass-stoppered bottles should be used instead of bottles with cork or rubber stoppers.)

Cholesterinization. A given amount of the alcoholic extract, sufficient for about two months' use, is measured into an Erlenmeyer flask; 6 mgm. of cholesterin of high purity are added per cubic centimeter. The flask is placed in a warm water bath and rotated to facilitate solution of the cholesterin. When the latter is entirely dissolved, the antigen is filtered and allowed to stand 1 day at room temperature. It is then ready to be titrated.

² In our experience alcoholic (cholesterin-free) extract keeps for many months although we have not had occasion to employ an antigen a year or more after preparation. The custom in this laboratory is to cholesterinize an amount of extract sufficient for about 2 months' use. Our experience therefore with the keeping quality of cholesterinized extract is limited to this period.

For use in the test, the antigen is diluted with salt solution according to its titre which is determined by the titration method discussed in the next chapter.

A summary of the steps in the preparation of antigen is presented in table 34.

TABLE 34

Outline of method for preparing antigen from powdered beef heart

Ether extrac- tion.	(1) 25 grams powder in 250 cc. Erlenmeyer—100 cc. ether added. Flask shaken 10 minutes to keep powder in suspension. Mixture filtered. Gentle pressure exerted by means of spatula until practically no ether drops from funnel. (2) Moist powder returned to flask—75 cc. ether added. Mixture shaken 10 minutes and filtered as in (1). (3) Moist powder returned to flask—75 cc. ether added. Mixture shaken 10 minutes and filtered as in (1). (4) Moist powder returned to flask—75 cc. ether added. Mixture shaken 10 minutes and filtered as in (1). Powder dried until free from ether odor.
Alcohol extrac- tion	Powder weighed and returned to flask used above (after complete drying). 5 cc. alcohol (95 per cent) added per gram of powder. Mixture shaken 10 minutes. Extracted 3 days at room temperature (21°C.) without shaking. Mixture shaken 5 minutes before filtration. Filtered extract kept in dark at room temperature as stock solution.
Addition of cholesterin	6 mgm. cholesterin added per cubic centimeter alcoholic extract. Cholesterinized extract filtered. Antigen now ready for titration. (Cholesterin is added to an amount of alcoholic extract sufficient for several months' use.)

SERUM

The major requirement regarding serum is that it be free from visible particles of any kind. This can be readily accomplished by thorough centrifugation of the blood to be

tested and subsequently pipetting off the supernatant serum with due care. Chylous sera or partially hemolyzed ones do not give any difficulty with this test. Practically all our studies with this test are based on blood reaching this laboratory two to three days after withdrawal. This would indicate that the age of sera, within certain limits, does not affect the reaction.

Blood is obtained in the usual manner from the median basilic vein, under aseptic conditions. The tube of blood is slanted and left at room temperature for half an hour to permit coagulation. The specimen is then sent to the laboratory where it is immediately placed in the refrigerator until time for examination. After thorough centrifugation, the serum is pipetted off and inactivated for 30 minutes at 56°C. when it is ready to be tested. The serum is preferably removed from the clot on the same day the tests are run. This, in our opinion, helps prevent contamination. If required to re-test a serum, it is re-inactivated for 10 minutes at 56°C.

Hemolysis of blood specimens may be avoided by taking proper precautions. Some frequent causes of hemolysis are: contamination; water in the syringe; discharging blood into container with force sufficient to break corpuscles; exposure to extremely high or low temperature incident to the weather; disturbing blood during the clotting period.

PHYSIOLOGIC SALT SOLUTION

The salt solution is prepared by adding 8.5 grams of chemically pure sodium chloride per 1000 cc. of distilled water. Chemical cleanliness rather than sterility is essential in the preparation of this solution.

CHAPTER XVII

TITRATION OF ANTIGEN FOR TEST WITH SERUM

Purpose. The purpose of the antigen titration is to determine the proper proportions of antigen and salt solution to employ in the preparation of the standard antigen dilution for the tests. Technically, it is to determine the minimum amount of salt solution which, when mixed with antigen, will produce a precipitate capable of readily dissolving on further addition of salt solution.

Procedure. 1. Add 1 cc. of cholesterinized antigen to each of 5 standard antigen dilution tubes (5.5 cm. length, 1.5 cm. diameter).

2. Add to 5 similar tubes 0.8, 0.9, 1.0, 1.1, 1.2, cc. physiologic salt solution, respectively.

3. Prepare 5 antigen dilutions by mixing the 1 cc. quantities of antigen with the varying amounts of salt solution, in series. Mix the salt solution with the antigen in each case by pouring the salt solution into the antigen as rapidly as possible (without waiting to drain the tube) and pouring the mixture back and forth 6 times.

4. Permit the antigen-saline dilutions to stand for 30 minutes.

5. Test the solubility in salt solution of each of the precipitates present in the antigen-saline dilutions—after thorough mixing—as follows:

a. Set up 3 standard tubes employed in performing the regular test with serum (7.5 cm. length, 1 cm. diameter).

b. Pipette 0.05, 0.025 and 0.0125 cc. quantities, respectively, of a given antigen dilution to the bottom of the tubes, using a 0.2 cc. pipette marked in 0.001 cc.

c. With a 1 cc. pipette, add 0.15 cc. physiologic salt solution to each tube.

d. Shake the tubes vigorously for 2 minutes.

e. Add 0.5 cc. salt solution to each tube and observe whether fluids are opalescent or contain precipitates.¹

Interpretation of titration results. When each of the 5 antigen dilutions are thus tested for the solubility of the precipitates by means of the 3 tube test, it will usually be found that the antigen dilutions prepared by mixing 1 cc. antigen and 0.8 or 0.9 cc. salt solution contain precipitates which are insoluble in salt solution, whereas the 3 remaining antigen dilutions, each containing 1 cc. amounts of antigen and 1, 1.1 and 1.2 cc. salt solution, respectively, contain precipitates which are soluble in salt solution. The antigen dilution containing the smallest amount of salt solution having a precipitate which is soluble in salt solution—as indicated by the 3 tube test—represents the titre of the antigen. If, for example, the 3 tube test indicates that 1 cc. amounts of antigen diluted with either 1, 1.1 or 1.2 cc. salt solution contain precipitates which are soluble in salt solution, then the titre of the antigen is $1 + 1$; that is, 1 cc. antigen is diluted for the tests with 1 cc. salt solution or 2 cc. antigen with 2 cc. salt solution.

Although we have not observed any changes in the titre of antigen on standing, it is well to retitrate from time to time.

Although non-syphilitic sera may safely be used for the purpose of titrating antigens, there are several advantages in employing salt solution. The latter being readily available, repetition is facilitated in the hands of workers inexperienced with the method. The use of salt solution, furthermore, permits an ample factor of safety in antigen dilution. Thus, a precipitate from a given antigen dilution which is soluble in salt solution is invariably soluble in serum.

Table 35 gives an outline of a typical antigen titration.

¹ Lately we observed that adding 1 cc. (instead of 0.5 cc.) salt solution to the tube containing 0.05 cc. antigen dilution and 0.15 cc. salt solution makes reading slightly easier.

TABLE 35
Typical antigen titration
 (To determine standard antigen dilution for use in test with serum)

	ANTIGEN DILUTION SERIES				
	1	2	3	4	5
Antigen + salt solution, cc.....	1 + 0.8	1 + 0.9	1 + 1.0	1 + 1.1	1 + 1.2
Result of dilution.....	Heavy precipitate in each antigen dilution				
Scheme used in testing solubility of precipitate in each antigen dilution	Tube number..... 1 2 3 *Antigen dilution cc..... 0.05 0.025 0.0125 Salt solution, cc..... 0.15 0.15 0.15 Tubes are shaken 2 minutes and 0.5 cc. salt solution added to each. All are observed for precipitates.				
Solubility of precipitate as determined by three-tube test	Precipitate not soluble	Precipitate not soluble	Precipitate soluble	Precipitate soluble	Precipitate soluble
Standard antigen dilution			Antigen + minimum amount of salt solution giving precipitate which dissolves in salt solution		

* Each antigen dilution is allowed to stand 30 minutes after mixing antigen and salt solution before solubility test is made.

CHAPTER XVIII

ROUTINE TEST WITH SERUM

Having presented the method for the titration of antigen, we are now ready to consider the test proper.

Since the standard antigen dilution is to be used within 10 to 30 minutes after mixing the antigen with salt solution, it is important to have the sera for any given number of tests inactivated and ready for pipetting and the tubes set up and numbered before preparing the antigen dilution.

Preparation of standard antigen dilution. 1. Add 1 cc. antigen to a standard antigen dilution tube (5.5 cm. length, 1.5 cm. diameter).

2. Add the amount of physiologic salt solution indicated by titration to a similar tube. (Titre of antigen is usually 1 cc. antigen + 1 cc. salt solution.) The same tubes may be used for mixing 2 cc. of antigen with salt solution.

3. Pour the salt solution into the antigen, and as rapidly as possible (without waiting to drain the tube) pour the mixture back and forth 6 times.¹

4. Allow this standard antigen dilution to stand 10 minutes before using and employ in the tests within a half hour after diluting the antigen with salt solution.

5. As a check on the proper mixing of antigen and salt solution, measure 0.05 cc. of the standard antigen dilution into a tube and add 1 cc. salt solution. After shaking vigorously for 15 seconds the mixture should appear opalescent.

¹ We have observed that beginners are often hesitant during the mixing process. The result is the appearance of cholesterol crystals in the antigen dilution soon after mixing the antigen with salt solution.

One cubic centimeter of antigen will be sufficient for about 15 tests with controls. In special cases 0.7, 0.8 or 0.9 cc. antigen may be mixed with the required amount of salt solution, also fractions above 1 cc., such as 1.1, 1.2, 1.3 cc., etc. In this laboratory where over 200 tests are carried out daily, 2.5 cc. antigen—sufficient for 40 tests—are usually prepared at one time. This can be pipetted by an experienced worker in about 8 minutes without any undue haste. As the pipetting of antigen dilution in a rack of tubes is completed by one worker, the rack is turned over to another worker who pipettes the serum. In following this procedure, 40 tests can be completed leisurely within 20 minutes.

Performance of test. 1. Measure 0.05, 0.025 and 0.0125 cc. quantities, respectively, of antigen dilution into 3 tubes (7.5 cm. length, 1 cm. diameter), with a 0.2 cc. pipette marked in 0.001 cc. Deliver to the bottom of the tubes.

2. Add 0.15 cc. amounts of clear inactivated (30 minutes at 56°C.) serum to each of the 3 tubes with a 1 cc. pipette graduated in 0.01 cc. Mix the antigen dilution and serum thoroughly.

3. Shake tests vigorously (preferably in shaking machine) for 2 minutes.

4. Add 0.5 cc. salt solution to each tube. (Some workers prefer adding 1 cc. instead of 0.5 cc. salt solution to the first tube of the test which contains the largest quantity of antigen dilution). The addition of salt solution to the tests clears the negatives and disperses the precipitates in the positives, rendering the reading of results easier. After the addition of salt solution to each tube, the tests are shaken for several seconds to permit thorough mixing and for uniformity are permitted to stand for 5 minutes at room temperature before making the reading of results.

Effect of incubation. Although the final results may be read immediately after the shaking period, it has been ob-

served that a 15 minute incubation period in the water bath at 37°C. produces sufficient clumping of the precipitates to make the reading of the results easier, particularly in the case of weak reactions. In studying the effect of 15 minutes incubation on this test, no tendency for false positive reactions has been observed, and on the basis of easier reading we consider the employment of this incubation period of some advantage. If employed, such incubation is always carried out after the 2 minute shaking period and prior to the addition of 0.5 cc. salt solution to each tube. If the tests are incubated after the addition of salt solution, an occasional weaker precipitate will go back into solution.

The reading of results. No rule can be made regarding the reading of results. Individual workers will be guided by their experience and by the special conditions under which they work. In this laboratory, the results are read in front of a window with a darkened background. The negative and the strongly positive reactions are read without removing the tubes from the rack, the negatives being opalescent and the positives showing heavy precipitates. Tubes showing questionable reactions are removed from rack and examined individually. The fluid in such a tube is spread out into a thin layer by slanting the tube in an almost horizontal position. The tube is raised some inches above the eye level and observed for the presence of a precipitate (plate IV).

Readings are recorded as four, three, two and one plus and doubtful on the basis of the distinctness of the precipitate. A four plus represents a complete precipitation reaction—the precipitate being suspended in a clear medium. A precipitate which borders on the four plus is read three plus. When a precipitate, although definite, gives the appearance of being too weak for a three plus, it is read

two plus. Correspondingly weaker precipitates are read one plus and doubtful, respectively.¹

In the reading of four plus reactions, it should be noted that the quantity of precipitate varies somewhat in the 3 tubes, due to the different amounts of antigen dilution used. The criterion in reading the results is based on the relative completeness of precipitation rather than the quantity of precipitate.

Whenever possible, the results should be read independently by two workers. When an extra worker is not available, the results should be re-read by the same worker about 10 minutes after the first reading.

Controls for each series of tests. 1. *Antigen control.* When pipetting antigen dilution for a series of tests, use the last set-up of 0.05, 0.025, and 0.0125 cc. amounts as controls—adding 0.15 cc. salt solution to each instead of serum. Include with the regular tests. All 3 tubes should show freedom from a precipitate.

2. *Serum control.* Examine each serum for particles which might give the appearance of a specific precipitate. Particularly in the case of positive reactions, it is essential to determine that the serum used in the test was entirely clear. In such reactions it is well to dilute the serum with

¹ Recently the author discussed nomenclature of serologic tests for syphilis with Professor Thorvald Madsen, Chairman of the League of Nation's Health Committee. Professor Madsen indicated the importance of employing a uniform method of reporting results by workers throughout the world. He further stated that the special committee which is studying serologic tests favored the employment of a ++, +, ±, — nomenclature.

In adapting this nomenclature to the author's routine test with serum, the following scheme is recommended:

Average result in author's 8-tube test	Nomenclature of League of Nation's Health Committee
++++ or ++++	is interpreted as ++
++	is interpreted as +
+ or ±	is interpreted as ±
—	is interpreted as —

The same scheme is applicable to the author's test with spinal fluids.

salt solution to approximately correspond with the final dilution of serum when the tests are read. This dilution of the serum occasionally renders visible fine particles which are invisible in undiluted serum.

3. *Positive and negative controls.* Include one or more such controls with each series of tests.

Interpretation of results. In attempting to interpret the results of this test, our guide has been the combined findings of two Wassermann tests—one carried out with a cholesterolized antigen and 1 hour fixation, and the other with a cholesterolin-free antigen and 4 hours fixation. These

TABLE 36
Control system for test with serum

POSITIVE SERUM CONTROL			NEGATIVE SERUM CONTROL			ANTIGEN CONTROL			SERUM CONTROL
Tube number	Standard antigen dilution	Serum undiluted	Tube number	Standard antigen dilution	Serum undiluted	Tube number	Standard antigen dilution	Salt solution	
	cc.	cc.		cc.	cc.		cc.	cc.	
1	0.05	0.15	4	0.05	0.15	7	0.05	0.15	Each serum is examined to establish freedom from particles
2	0.025	0.15	5	0.025	0.15	8	0.025	0.15	
3	0.0125	0.15	6	0.0125	0.15	9	0.0125	0.15	

are considered conservative tests compared with those employing fixation periods of about 16 hours and are the routine tests employed in this laboratory. It was observed in interpreting the degree of precipitation on a plus-sign basis that the average of the findings of the 3 tubes approximated the findings with the Wassermann tests. It is recognized that only clinical studies can be the true guide in correctly interpreting a diagnostic test. In view, however, of the wide acceptance of the Wassermann test in connection with the diagnosis of syphilis, it was believed important to correlate the findings of the precipitation test with

those of the Wassermann. Such correlation, furthermore, would render uniform the methods of reporting both tests to physicians as well as unify the interpretation of results. The average finding of the 3 tubes has therefore been adopted

TABLE 37
Outline of test and interpretation of results

		THE TEST			
		Tube 1	Tube 2	Tube 3	Completion of test
Serum:antigen dilution.....		3:1	6:1	12:1	Shake tests two minutes. Add 0.5 cc. salt solution to each tube and read results
Antigen dilution, cc.....		0.05	0.025	0.0125	
Serum (heated at 56°C. for 30 minutes), cc.....		0.15	0.15	0.15	
		Interpretation of results			
	Reaction number				Average of reactions of three tubes (final results)
Some typical reactions	1	++++	++++	++++	++++
	2	+++	++++	++++	
	3	++	++++	++++	+++
	4	+	+++	++++	
	5	—	+++	++++	++
	6	—	++	++++	
	7	—	±	++++	+
	8	—	—	+++	
	9	—	±	++	±
	10	—	—	—	—

as representing the final result of a given test. Table 37 gives an outline of some typical reactions with the scheme of interpretation.

It is urged that, in reporting the results of the routine test to physicians, the findings in the 3 individual tubes be given in addition to the average findings. The physician



(Photo by L. C. Hulbert, Mich. Dept. of Health)

READING RESULTS OF AUTHOR'S TEST

Readings are made in front of a partly shaded window with a darkened background. The strongly positive sera show heavy precipitates which are readily distinguishable without lifting the tubes from the rack. In the case of the weakly positive sera, each tube is examined as illustrated.

would then have not only a result comparable with a conservative Wassermann, but a more quantitative indication of the serologic condition of the patient. It should be remembered, however, that only the average result should be considered as a means of diagnosis of syphilis.

The test with large numbers of specimens. The following suggestions are made with a view of aiding workers who are called upon to examine large numbers of sera at a given time.

Two workers should be available, one to pipette antigen dilution and the other, serum. Everything should be in readiness at the time planned for the tests. The sera should be inactivated, the tubes set up and numbered and suitable pipettes ready for use.

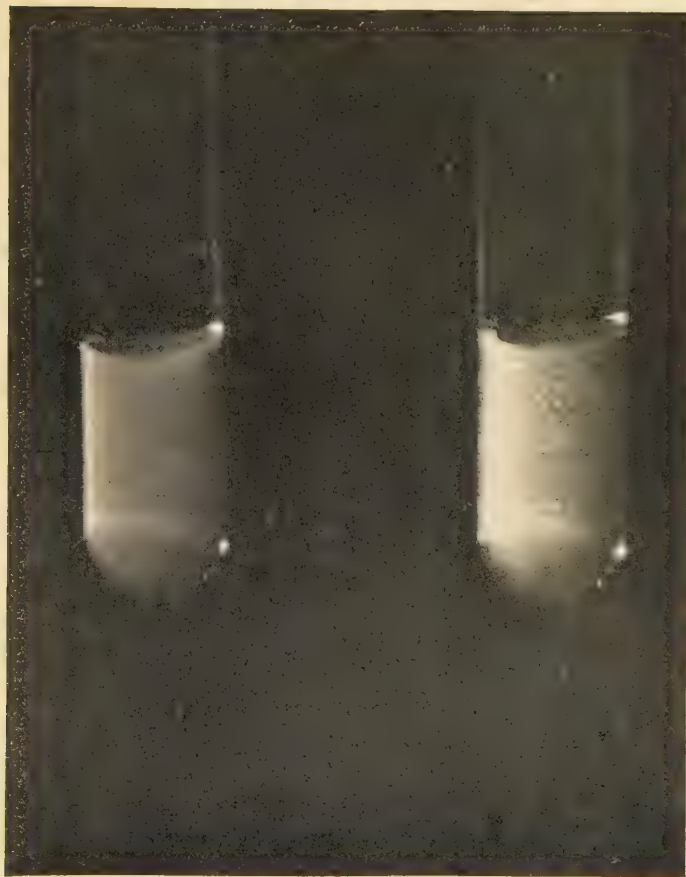
It is well to use three different pipettes for measuring antigen dilution. The 0.05 cc. amounts are pipetted with a 1 cc. pipette on which 0.05 cc. graduations are indicated with a wax pencil. The 0.025 and 0.0125 cc. amounts of antigen dilution are pipetted with two 0.2 cc. pipettes and the proper markings on each pipette may also be indicated with a wax pencil. The standard rack contains three rows of tubes. In the first row are pipetted 0.05 cc. amounts of antigen dilution, and in the second and third 0.025 and 0.0125 cc. amounts of antigen dilution, respectively. Each of the three antigen dilution pipettes, after it has been used, is kept in a test tube containing some salt solution until its use for the next rack. This prevents the drying of the antigen dilution which adheres to the tip of the pipettes. The trace of saline in the pipettes is thoroughly removed before again inserting the pipettes into the antigen dilution. In this connection it should be pointed out that it is important to mix the antigen dilution frequently during the pipetting period to assure a uniform mixture.

In planning for the tests, workers should bear in mind that, after the antigen is diluted with salt solution, the anti-

gen dilution is allowed to stand for 10 minutes before pipetting and it is mixed with sera for the tests within 30 minutes. This leaves but 20 minutes for pipetting the antigen dilution and sera. Antigen dilution for 40 tests (2.5 cc. standard antigen mixed with the proper amount of salt solution) can be pipetted easily in from 5 to 8 minutes. Twenty minutes, therefore, is ample time for pipetting both antigen dilution and sera for 40 tests. For 80 tests, two quantities of antigen dilution, each consisting of 2.5 cc. antigen diluted with the required amount of salt solution, may be prepared, the second quantity being prepared 10 minutes after the first. When the first quantity of antigen dilution has been pipetted, the second quantity will have stood for nearly 10 minutes and the worker can continue pipetting very soon, almost without interruption. When a still larger number of examinations are being made, the pipetting of antigen dilution can be arranged accordingly.

As soon as antigen dilution has been pipetted into a rack of tubes, the rack is given to another worker who is ready to pipette the sera (0.15 cc. of each serum into each of the three different quantities of antigen dilution, respectively). There should be no undue delay in adding the serum to the antigen dilution since the small quantities of the latter favor rapid evaporation which, in turn, might lead to incorrect results. As soon as the sera have been pipetted into one rack of tubes containing antigen dilution, the rack is shaken for about 15 seconds to assure thorough mixing of the ingredients. The rack may then be left at room temperature until the ingredients for the remainder of the tests have been pipetted and mixed. All the tests are then shaken for a period of two minutes. The use of a shaking machine is particularly desirable for large numbers of tests.

If, at any time, it is necessary for one worker to set up the tests alone, it is suggested that antigen dilution be pipetted for one rack of 10 tests at a time. This is followed directly



(Photo by L. C. Hulbert, Mich. Dept. of Health)

TUBES SHOWING POSITIVE AND NEGATIVE PRECIPITATION REACTIONS
IN AUTHOR'S TEST (ENLARGED)

with the sera for these tests. As already indicated, the ingredients are then thoroughly mixed and the rack set aside until the pipetting for the remainder of the tests is completed and all tests are ready for the two minute shaking period.

The test with non-standard quantities. It is occasionally desired to make a serologic test with a very small amount of serum. This is particularly true in the case of infants from whom the withdrawal of blood is frequently difficult. Under these conditions, there is no reason why a precipitation test might not be run with 0.1 or even 0.05 cc. of serum. When inactivating small amounts, the tubes should be corked to prevent evaporation. A 1 tube test containing a 10:1 proportion of serum to antigen dilution is recommended. Thus, with 0.1 cc. of serum, 0.01 cc. of antigen dilution is employed; with 0.05 cc. of serum, 0.005 cc. of antigen dilution is used. The test is completed in the usual manner, shaking the tube for 2 minutes after adding the serum to the antigen dilution. Before reading, a small amount of salt solution, such as 0.2 cc., is added. In reporting such a test, the physician should be informed that non-standard amounts were employed.

The routine test is also adaptable to fluids obtained from syphilitic lesions. Such fluids, even when diluted in some measure with salt solution, have been found to give definite precipitation reactions. We have used in this case also a 10:1 proportion of fluid to antigen dilution. Such tests should be of particular value in the case of chancre fluid obtained in the early primary stage.

CHAPTER XIX

QUANTITATIVE PROCEDURE WITH SERUM

The use of 3 serum-antigen proportions renders the routine test quantitative to a degree, but not sufficiently so to satisfy special conditions. A serum from a case of secondary syphilis, for example, may continue to give four plus reactions with the routine test after several courses of treatment, whereas there may have been a considerable reduction of the reacting substances in the serum. It is with a view to satisfying such special conditions that this method is presented.

THE UNIT REACTION

The determination of a serum reacting unit was discussed in Chapter VI. Briefly, complete precipitation resulting from mixing 0.15 cc. serum with 0.0125 cc. standard antigen dilution was considered as 4 reacting units. By employing the amount of antigen dilution as a constant with varying dilutions of a given syphilitic serum with salt solution, the number of reacting units was determined according to the formula $S = 4 D$, where S represented the potency of the serum in terms of reacting units and D the maximum dilution giving a definite precipitate. This unit system was also employed in a discussion of the quantitative procedure published elsewhere.¹ After the development of the presumptive procedure (Chapter XXI), we had occasion to use the antigen of this procedure in a series of experiments with the quantitative method. In these experiments, all phases of the quantitative method were essentially the same as originally described except, that, instead of employing

¹ Amer. Jour. Pub. Health, 1924, XIV, 498.

0.0125 cc. of the standard antigen dilution with 0.15 cc. of serum as the basis of the unit system, 0.01 cc. of the special antigen dilution with 0.15 cc. of serum was employed. It was observed that the special antigen gave more sensitive reactions in the quantitative procedure than the standard antigen. This was considered a distinct advantage, since the quantitative method is applicable only to treated cases and sensitiveness of reactions is desirable. After using the special antigen in a large number of quantitative tests, it was adopted as the basis of the quantitative procedure which is described in detail in this chapter.

When 0.15 cc. of serum, in an undiluted form, mixed with 0.01 cc. of the special antigen dilution (presumptive procedure) results in a definite precipitation reaction, it is interpreted as a four unit reaction. By employing the antigen dilution as a constant with varying dilutions of serum with salt solution, the number of reacting units is computed according to the formula referred to above, $S = 4 D$. Thus, if 1:5 represents the highest dilution of serum capable of giving definite precipitation, the serum contains 4×5 or 20 reacting units. If 1:50 represents the highest dilution in which a serum gives a definite precipitation reaction, the serum contains 4×50 or 200 units. Similarly the potency of any serum may be determined.

It should be noted that only definite precipitation reactions are considered in determining the concentration of reacting units in a given serum. A reaction giving the appearance of one plus or doubtful is considered negative as in the presumptive procedure.

If desired, the potency of a given serum may also be expressed in reacting units per cubic centimeter. Since 0.15 cc. of serum is the amount employed in the quantitative procedure, this amount multiplied by $6\frac{2}{3}$ will give the number of units per cubic centimeter. Thus, if 1:30 represents the maximum dilution of a serum giving a com-

plete precipitation reaction, this serum contains 4×30 or 120 reacting units in 0.15 cc. or $6\frac{2}{3} \times 120 = 800$ reacting units in 1 cc. There is, however, no advantage in expressing the potency of sera in terms of units per cubic centimeter as the relative character of the findings is not affected. There is, on the other hand, an advantage in expressing the potency directly by means of the formula $S \times 4 D$ on the basis of simplicity.

PROCEDURE

The procedure outlined embraces 8 different serum dilutions. This series of dilutions has been found to include the highest dilution giving complete precipitation in the majority of syphilitic sera. The dilutions range from 1:1 to 1:60 with salt solution. They may be prepared as follows:

1:1	= undiluted serum
1:5	= 0.2 cc. undiluted serum + 0.8 cc. salt solution
1:10	= 0.6 cc. 1:5 dilution + 0.6 cc. salt solution
1:20	= 0.2 cc. 1:10 dilution + 0.2 cc. salt solution
1:30	= 0.2 cc. 1:10 dilution + 0.4 cc. salt solution
1:40	= 0.1 cc. 1:10 dilution + 0.3 cc. salt solution
1:50	= 0.1 cc. 1:10 dilution + 0.4 cc. salt solution
1:60	= 0.1 cc. 1:10 dilution + 0.5 cc. salt solution

Eight tubes are set up and 0.01 cc. special antigen dilution of presumptive procedure is pipetted into each in the usual manner.

The serum dilutions in 0.15 cc. quantities are now added, in order, to the tubes containing antigen dilution.

The rack is shaken for 3 minutes, 0.5 cc. salt solution added to each tube, and after 5 minutes the results are read and recorded.

If a 15 minute incubation period at 37°C. is employed, it should be carried out as suggested in connection with the regular test, namely, after the shaking period and prior to

TABLE 38
Some typical reactions with the quantitative procedure

Serum : salt solution.....	1:1	1:5	1:10	1:20	1:30	1:40	1:50	1:60	FOUR TIMES THE MAXIMUM DILUTION OF SERUM GIVING DEFINITE PRECIPITATION (4 D)	SERUM POTENCY IN TERMS OF REACTING UNITS (S)
Serum dilution, cc.....	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15		
Antigen dilution (special antigen), cc.....	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01		
Serum number										
1	Pos.*	—	—	—	—	—	—	—	4 × 1	4
2	Pos.	Pos.	—	—	—	—	—	—	4 × 5	20
3	Pos.	Pos.	Pos.	—	—	—	—	—	4 × 10	40
4	Pos.	Pos.	Pos.	Pos.	—	—	—	—	4 × 20	80
5	Pos.	Pos.	Pos.	Pos.	Pos.	—	—	—	4 × 30	120
6	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	—	—	4 × 40	160
7	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	Pos.	—	4 × 50	200

* Pos. = definite precipitation reaction.

the addition of salt solution. For details of the preparation and titration of the special antigen, the reader is referred to Chapter XXI.

The quantitative procedure, including the preparation of the serum dilutions, is carried out in duplicate. If necessary, 0.1 instead of 0.2 cc. of serum may be employed in the preparation of the serum dilution series.

Sera of unusually high potency giving definite precipitation in 1:60 dilution are diluted still further with salt solution until a negative reaction is obtained.

Some typical reactions with the Quantitative Procedure are illustrated in table 38.

The quantitative procedure is applicable only to sera giving strong reactions with the routine test. Sera giving weak reactions with the routine test give negative results after dilution with salt solution.

The results of the quantitative procedure are always expressed in terms of reacting units.

CHAPTER XX

PROCEDURE WITH SPINAL FLUID

There is perhaps no better practical illustration of the importance of concentration in the serology of syphilis by precipitation than the reactions obtained by this test with spinal fluids. It was early observed that only those fluids which contained large numbers of reacting substances gave precipitation reactions with antigen dilution, those containing comparatively small numbers being negative. This was undoubtedly due to the fact that the high dilution element of these fluids interfered with precipitation. The obvious solution to this difficulty was to concentrate the reacting elements of the spinal fluid, possibly by precipitating the proteins and subsequently dissolving them in a minimum amount of salt solution. To Herrold (31) belongs the credit for having succeeded in doing this in a manner applicable to our test. In attempting to apply the ring modification of the writer's test (32) to spinal fluids, Herrold suggested precipitating the globulins in these fluids with saturated ammonium sulphate solution and taking up the precipitate in a minimum amount of salt solution after centrifuging and pouring off the supernatant fluid.

In our attempts to precipitate the globulins of the spinal fluid, it was believed that the use of very weak acid for this purpose might be a safer procedure than the use of ammonium sulphate, having early observed that various concentrations of the latter salt are capable of giving definite precipitation with our standard antigen dilution. However, the use of weak acid as a globulin precipitant was soon found to have an important practical difficulty. As many as 30

cc. of weak acid per cubic centimeter of spinal fluid were necessary to throw down the globulins. The impracticability of centrifuging comparatively large volumes, particularly when employing several cubic centimeters of spinal fluid, led us to turn to ammonium sulphate as a possible means for concentrating the proteins in the fluid.

After some trial, it appeared to us that ammonium sulphate might be used with safety in conjunction with a spinal fluid precipitation method, provided every step of the procedure was carried out with care and properly controlled. We thereupon invited Dr. H. L. Keim, with whom we have been associated in clinical studies on this precipitation test, to investigate the clinical value of the spinal fluid procedure at the Dermatological Clinic of the University of Michigan Medical School. The favorable results obtained by Dr. Keim on fifty fluids with this procedure, combined with our own on an equal number, led us to make a preliminary report. We have now a record of over 750 examinations comparable with the Wassermann and, to our knowledge, with no record of falsely positive reactions.

TECHNIC

Preparation of concentrated globulin solution. 1. Centrifuge spinal fluid to remove cellular matter.

2. Measure 3 cc. of clear fluid into a centrifuge tube.

3. Add 2 cc. saturated solution of chemically pure ammonium sulphate, mix and permit to stand for 1 hour at room temperature.

4. Centrifuge at high speed for 15 minutes.¹

¹ In a small per cent of spinal fluids it is difficult to throw down the globulin precipitate even after prolonged centrifugation. In such cases a minute amount of talc is added and the mixture centrifuged for about 15 minutes. After pouring off the supernatant fluid as completely as possible, the precipitate is suspended in 0.3 cc. salt solution and this suspension centrifuged. The test is completed with the supernatant fluid which contains the dissolved globulin.

5. Pour off supernatant fluid. Remove last drop adhering to lip of tube by means of filter paper.

6. To the remaining precipitate, add 0.3 cc. salt solution (one-tenth of the original volume of spinal fluid), lowering the pipette close to the bottom of the tube to avoid washing down ammonium sulphate from the side. This concentrated globulin solution is ready to be tested with antigen dilution for specific reacting substances.

Preparation of standard antigen dilution. Prepare standard antigen dilution as described under routine test with serum, using the proportion of salt solution to antigen indicated by special titration method which follows. The antigen dilution is allowed to stand for 10 minutes before using—as in the test with serum.

Performance of test. 1. For each spinal fluid to be tested, deliver 0.01 cc. antigen dilution to the bottom of each of 2 tubes (7.5 cm. by 1 cm.) by means of a 0.2 cc. pipette.

2. To each tube add 0.15 cc. concentrated globulin solution prepared from the spinal fluid and mix with the antigen dilution.

3. Shake the tests vigorously for 3 minutes. (Incubation period of fifteen minutes at 37°C. may be employed as suggested under test with serum, if desired.)

4. Add 0.5 cc. salt solution to each tube and after 5 minutes read the reactions as in the test with serum. Record on the basis of + + + +, + + +, + +, +, ± and — depending on distinctness of the precipitate.

Controls. The control system is of particular importance in the spinal fluid test in view of the fact that certain concentrations of ammonium sulphate, as already indicated, are capable of giving precipitates with standard antigen dilution.

1. As a special control, mix 3 cc. salt solution with 2 cc. concentrated ammonium sulphate at the time the same quantities of spinal fluid and ammonium sulphate are

mixed. Pour off the fluid in the same manner that the spinal fluid-ammonium sulphate fluid is poured off after centrifugation and add 0.3 cc. salt solution. Mix 0.15 cc. of this solution with 0.01 cc. antigen dilution and include with the spinal fluid tests. When the tests are read this control tube should be free from a precipitate.

2. Positive and negative controls. Concentrate a positive and a negative spinal fluid by means of ammonium sulphate—as in the case of the fluid to be tested—and include tests of these fluids with antigen dilution in each series.

3. Concentrated fluid control. Examine each concentrated fluid for particles which might give the appearance of a specific precipitate.

Method of titrating antigen for use in test with spinal fluids. A special titration of the antigen is necessary for spinal fluids because of the use of ammonium sulphate for the concentration of the globulins. Since ammonium sulphate in sufficient concentration is capable of producing a precipitate with antigen dilution, it is essential that the titre of the antigen be determined by a titration method in which the solubility of the precipitate formed in the antigen dilution is tested in ammonium sulphate solution of approximately the same concentration as that of the globulin solution used in the test. The employment of this ammonium sulphate solution for titration leads to the use in the spinal fluid test of an antigen dilution containing slightly more salt solution than the antigen dilution for the serum test. Thus, if the standard titre of a given antigen with serum is 1 cc. of antigen plus 1 cc. saline, the titre of this antigen for spinal fluids will probably be 1 cc. antigen plus 1.3 or 1.4 cc. saline. This small increase in salt solution for antigen dilution appears to counterbalance the tendency for precipitation of the ammonium sulphate solution with the antigen dilution.

Titration procedure. 1. Measure five 1 cc. quantities of antigen into 5 standard antigen dilution tubes.

2. Into 5 similar tubes measure 1.1, 1.2, 1.3, 1.4 and 1.5 cc. quantities of physiologic salt solution, respectively.

3. In making the 5 antigen dilutions, pour the salt solution into the antigen in each case and immediately pour the mixture back and forth 6 times.

4. After allowing these antigen dilutions to stand for 20 minutes, test the solubility of the precipitate present in each as follows:

a. Prepare a solution of approximately 10 per cent ammonium sulphate by mixing 1 cc. saturated ammonium sulphate solution and 9 cc. salt solution.

b. Measure 0.01 cc. of each antigen dilution into each of 2 tubes.

c. To each tube add 0.15 cc. of the approximately 10 per cent ammonium sulphate solution.

d. Shake tests vigorously for 3 minutes.

e. Add 0.5 cc. salt solution to each tube and examine for precipitates.

5. The titre of the antigen is represented by the dilution of antigen containing the least salt solution, in which the precipitate is soluble in the ammonium sulphate solution. For example, if the dilutions 1 + 1.1 and 1 + 1.2 show precipitates which are insoluble in the ammonium sulphate solution, while 1 + 1.3 and 1 + 1.4 show precipitates which are soluble in the ammonium sulphate solution, the titre of the antigen is 1 + 1.3; that is, 1 cc. of antigen is diluted with 1.3 cc. salt solution.

Table 39 gives a typical titration of antigen for test with spinal fluids.

Employment of special antigen in spinal fluid test. Since the development of the spinal fluid test as described herewith, the perfection of the presumptive procedure (Chapter XXI) was brought about. Experiments were thereupon carried out with a view to determining whether the special antigen used in this procedure could be used in the spinal

TABLE 39
Typical antigen titration
 To determine antigen dilution for use in test with spinal fluid

	ANTIGEN DILUTION SERIES				
	1	2	3	4	5
Antigen + salt solution, cc.....	1 + 1.1	1 + 1.2	1 + 1.3	1 + 1.4	1 + 1.5
Result of dilution.....	Heavy precipitate in each antigen dilution				
Scheme used in testing solubility of precipitate in each antigen dilution	Tube number..... 1 2 *Antigen dilution, cc..... 0.01 0.01 10 per cent solution ammonium sulphate, cc..... 0.15 0.15 Tubes are shaken 3 minutes and 0.5 cc. salt solution added to each. All are observed for precipitates.				
Solubility of precipitate	Precipitate not soluble	Precipitate not soluble	Precipitate not soluble	Precipitate soluble	Precipitate soluble
Standard antigen dilution				Antigen + minimum amount of normal saline giving precipitate which dissolves in 10 per cent ammonium sulphate solution	

* Each antigen dilution is allowed to stand 20 minutes after mixing antigen and salt solution before solubility test is made.

fluid test. It was observed that the antigen titration scheme presented in this chapter was readily applicable to the special antigen. The only change required in this titration was to slightly increase the amount of physiologic salt solution with which to dilute the antigen. Thus, whereas in the titration of standard antigen 1 cc. amounts are mixed with 1.1, 1.2, 1.3, 1.4 and 1.5 cc. amounts of salt solution, respectively (table 39), in the titration of the special antigen, 1.4, 1.5, 1.6, 1.7 and 1.8 cc. amounts of salt solution are employed. The end-point of the titration with the special antigen is the same as with the standard antigen, namely, the smallest amount of physiologic salt solution mixed with antigen resulting in a precipitate which readily dissolves in an approximately 10 per cent ammonium sulphate solution. The usual titre of the special antigen is 1 cc. + 1.5 or 1.6 cc. physiologic salt solution.

Instead of employing two 0.01 cc. amounts of the standard antigen dilution in the spinal fluid test, we recently had occasion to substitute one 0.01 cc. amount with the special antigen dilution. The results consistently indicated a somewhat higher degree of sensitiveness with the special antigen dilution. Furthermore, no false reactions have been observed with this antigen dilution.

In accordance with this observation, the routine procedure with spinal fluids employed in this laboratory consists of a set-up of two tubes, one containing 0.01 cc. standard antigen dilution and the other 0.01 cc. special antigen dilution. Both tubes then receive 0.15 cc. concentrated globulin solution and the results are read after the 3-minute shaking period as outlined above.

The final result in the tube containing standard antigen compares with the result of a conservative Wassermann test and is considered of value particularly in diagnosis of neurosyphilis. The final result in the special antigen tube is more sensitive than a conservative Wassermann test and is considered of value particularly as a check on treatment.

CHAPTER XXI

PRESUMPTIVE PROCEDURE OF HIGH SENSITIVENESS¹

Studies on the factors governing the sensitiveness of antigen, as well as those governing the quantitative relation between serum and antigen and the period of shaking or agitation, led to the evolution of this presumptive procedure (Chapter XIV).

PREPARATION OF ANTIGEN

Ether extraction. 1. Place 25 grams of powdered beef heart into a 250 cc. Erlenmeyer flask.

2. Add 50 cc. ether; mix and shake flask from time to time for 5 minutes.

3. Filter. Apply gentle pressure to the beef heart in the funnel by means of a spatula. Filtration is completed when further pressure does not cause drops of ether to pass through the funnel.

4. Transfer the beef heart powder from the funnel to the original extraction flask.

5. Add 50 cc. of ether and shake from time to time for a period of 5 minutes.

6. Filter as in 3 and transfer the powder into the extraction flask.

7. Add 40 cc. of ether and shake from time to time for a 5 minute interval.

8. Filter as in 3; transfer the powder to extraction flask; add 40 cc. of ether and shake for a 5 minute period.

9. Filter as in 3, employing a fresh layer of filter paper. This completes the ether extraction.

¹ For want of a better term, "presumptive procedure" was chosen—on the basis of the presumptive character of the results.

10. Dry the beef heart by spreading on a glass plate or clean sheet of paper and placing in the incubator at 37°C. for about 15 minutes or at room temperature for a somewhat longer period. Drying is completed when the powder has no ether odor. The powder is then ready for the alcohol extraction.

Alcohol extraction. 1. Reweigh the powder and place it in the original ether extraction flask after removal of traces of ether by suction.

2. Add 5 cc. of 95 per cent alcohol per gram of powder.

3. Shake for 10 minutes to assure thorough mixing.

4. Extract for 3 days at room temperature without shaking the flask.

5. Shake flask for 5 minutes after the extraction period and filter.

6. Store filtrate in the dark at room temperature as stock solution. If a precipitate should appear after some months' standing, rotate flask in a 56°C. water bath for about 5 minutes and the precipitate will be found to re-dissolve.

Cholesterinization. 1. Measure a quantity of alcoholic extract, sufficient for several months' use, into a flask and add 6 mgm. cholesterin per cubic centimeter.

2. Dissolve the cholesterin by gently rotating the flask in a water bath.

3. Filter and permit the extract to stand 24 hours at room temperature. The antigen is then ready to be titrated.

TITRATION OF ANTIGEN

1. Add 1 cc. of the cholesterinized antigen to each of 5 standard antigen dilution tubes.

2. Add to five similar tubes 1, 1.1, 1.2, 1.3 and 1.4 cc. physiologic salt solution, respectively.

3. Prepare 5 antigen dilutions by mixing the 1 cc. quantities of antigen with the varying amounts of salt solution,

in series. Mix the salt solution with the antigen in each case by pouring the antigen into the salt solution as rapidly as possible and (without waiting to drain the tube) pouring the mixture back and forth 6 times.

4. Permit the antigen-saline dilutions to stand for 30 minutes at room temperature.

5. Test the solubility in salt solution of each of the precipitates present in the antigen-saline dilutions—after thorough mixing—as follows:

a. Set up 2 standard tubes employed in performing the regular test with serum (7.5 cm. length, 1 cm. diameter).

b. Pipette 0.01 cc. quantities, respectively, of a given antigen dilution to the bottom of the tubes, using a 0.2 cc. pipette marked in 0.001 cc.

c. With a 1 cc. pipette, add 0.15 cc. physiologic salt solution to each tube.

d. Shake the tubes for 3 minutes.

e. Add 0.5 cc. salt solution to each tube and observe if fluids are opalescent or contain precipitates.

Outline of procedure for determining whether or not precipitate in each antigen dilution is soluble

	TUBE 1	TUBE 2
Antigen dilution, cc.....	0.01	0.01
Normal saline, cc.....	0.15	0.15

Tubes are shaken vigorously for 3 minutes, 0.5 cc. saline added to each tube and observed for precipitates.

Interpretation of titration results. When each of the 5 antigen dilutions are thus tested for the solubility of the precipitates, it is usually found that the antigen dilutions prepared by mixing 1 cc. antigen and 1 or 1.1 cc. salt solution contain precipitates which are insoluble in salt solution, while the 3 remaining antigen dilutions, each containing 1 cc. amounts of antigen and 1.2, 1.3 and 1.4 cc. salt solution, respectively, contain precipitates which

are soluble in salt solution. The antigen dilution containing the smallest amount of salt solution having a precipitate which is soluble in salt solution—as indicated by the solubility test—represents the titre of the antigen. If, for example, the test indicates that 1 cc. amounts of antigen diluted with either 1.2, 1.3, or 1.4 cc. salt solution contain precipitates which are soluble in salt solution, then the titre of the antigen is $1 + 1.2$; that is, 1 cc. antigen is diluted for the tests with 1.2 cc. salt solution.

PERFORMANCE OF TEST

Preparation of antigen dilution. 1. Add 1 cc. of the special antigen to a standard antigen dilution tube.

2. Add an amount of salt solution indicated by titration to a similar tube.

3. Pour the salt solution into the antigen and, as rapidly as possible, pour the mixture back and forth 6 times.

4. Allow this antigen dilution to stand 10 minutes at room temperature before using.

The test proper. 1. Pipette 0.01 cc. of antigen dilution into a standard tube (7.5 cm. length, 1 cm. diameter), with a 0.2 cc. pipette marked in 0.001 cc.—delivering to the bottom of the tube.

2. Add 0.15 cc. clear inactivated (30 minutes at 56°C.) serum with a 1 cc. pipette graduated in 0.01 cc. Mix the antigen dilution and serum thoroughly.

3. Shake test vigorously (preferably in shaking machine) for 3 minutes.

4. Add 0.5 cc. salt solution to tube and after 5 minutes observe for precipitate. Readings are made in front of a window with a darkened background. The negative and positive reactions may be read in practically all cases without removing the tubes from the racks. In the case of questionable reactions, lift each tube from rack considerably above the eye level—slanting to spread the fluid into a thin layer—and examine for a precipitate.

Control system. 1. *Antigen control.* When pipetting antigen dilution for a series of tests, use the last tube containing 0.01 cc. with salt solution instead of serum and include with the regular tests. At the final reading, this tube should show freedom from a precipitate.

2. *Serum control.* Examine each serum for particles which might give the appearance of a specific precipitate. Particularly in the case of each positive reaction, it is essential to determine that the serum used in the test

TABLE 40
Outline of presumptive procedure

TUBE NUMBER	ANTI- GEN DILU- TION	SERUM	SALT SOLU- TION	COMPLETION OF TEST
	cc.	cc.	cc.	
Tube 1, test proper....	0.01	0.15		Tubes are shaken for 3 minutes; 0.5 cc. salt solution added to each and all tubes observed for precipitates
Tube 2, antigen dilution control.....	0.01		0.15	
Tube 3, positive serum control.....	0.01	0.15		
Tube 4, negative serum control.....	0.01	0.15		
Serum control.....		Each serum examined to establish freedom from particles		

was entirely clear. When using the presumptive test as a check on the routine test, the same serum control is sufficient for both tests.

3. *Positive and negative controls.* Include one or more such controls in each series of tests.

Table 40 gives an outline of the presumptive procedure.

Interpretation of results. The results in this procedure are read as positive or negative. Tests showing precipitates which appear distinct and unmistakable are read positive; all others are read negative.

PART V
CLINICAL APPLICATION

CHAPTER XXII

ONE HUNDRED THOUSAND EXAMINATIONS COMPARED WITH THE WASSERMANN TEST

Preliminary studies on the early procedure of the test completed by November 29, 1921, were sufficiently promising to begin on that date a daily tabulation of comparative findings with the Wassermann. By July 1, 1922, results with over 15,000 comparative tests were recorded. These findings indicated that the early procedure approached a high degree of practicability and led Dr. C. C. Young, director of these laboratories, to add this test to the various diagnostic procedures and to begin reporting the results to physicians parallel with the Wassermann test. This, to our knowledge, was the first attempt on the part of a public health laboratory to utilize a precipitation test for syphilis as a diagnostic method. The value of this method is illustrated by the studies of Keim and Wile (33), Herrold (32), Young (34), Ide and Smith (35), Levin (36), Mannheims (37), Babonneix, Boucher and Choay (38), Grant (39), Dulaney (40), Holmes (41), Strumia (42), Litterer (43), Yagle and Kolmer (44), Detweiler (45), Anderson and Fisher (46), Fox and Sanderson (47), Elliott and Todd (48), Havens and Taylor (49), Keim (50), Malcolm (51), Ishii (52), and Rockstraw and Bent (53).

Further studies on the precipitation phenomenon in syphilis led to the evolvement of the present procedure and on May 15, 1923, this procedure was substituted in the routine work in place of the earlier procedure. Studies on the standardized procedure have been reported by Rothberg (54), Osmond and McClean (55), Rockstraw and Hopkins (56), Wilson and Nedley (57), and Kendrick (29).

The Wassermann test as carried out in this laboratory consists of a sheep-cell-guinea-pig complement system. It is employed in duplicate with a cholesterinized antigen in one case and a cholesterin-free antigen in the other.

TABLE 41

Comparative findings of Wassermann and Kahn tests with 101,200 sera

(From November, 1921, to August, 1924)

	KAHN		WASSERMANN		
	Reaction*	Number of examinations	Positive	Doubtful	Negative
Results based on earlier procedure.....	Positive	8,120	7,549	395	176
	Doubtful	2,606	143	1,629	834
	Negative	32,304	143	816	31,345
Results based on present procedure.....	Positive	10,821	10,414	309	98
	Doubtful	2,001	25	1,259	717
	Negative	45,348	21	200	45,127

Per cent check† with Wassermann

	TOTAL EXAMINATIONS	ABSOLUTE CHECK	RELATIVE CHECK	NO CHECK
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Earlier procedure.....	43,030	94.17	5.08	0.74
Present procedure.....	58,170	97.64	2.15	0.21

* Positive reaction = +++++, +++ and ++.

Doubtful reaction = + and ±.

† Absolute check = positive or negative by both methods.

Relative check = positive or negative by one method and doubtful with the other.

The principles upon which our Wassermann technic is based are fully discussed elsewhere (58).

Table 41 gives the comparative results with both the earlier and present precipitation procedures and the Wassermann test. The data for this table was obtained from the official laboratory records.

OUTLINE OF THE ROUTINE USE OF AUTHOR'S TEST IN THE
MICHIGAN STATE LABORATORIES

The following outline presents, in a general way, the daily routine procedure in making about 200 examinations with the author's test, parallel with the Wassermann, in the Bureau of Laboratories of the Michigan Department of Health.

Approximately 150 blood specimens arrive in the morning mails and about 50 in the afternoon. On a given day precipitation tests are made on all specimens received in the forenoon and those received the previous afternoon. By 10:30 in the morning, about 120 of these specimens have been centrifuged; the sera pipetted off and inactivated for 30 minutes at 56°C. By 12:00 o'clock this number has been examined with the precipitation test and recorded. The remaining specimens, about 80, are examined between 2:30 and 4:00 in the afternoon. This period, as well as the interval between 10:30 and 12:00 in the morning, is chosen for the test because of convenience in relation to the Wassermann routine.

All the sera examined by the author's test on a given day are reactivated for 5 minutes the following morning and examined with the Wassermann test. When these tests are completed, the results are compared with the precipitation findings recorded the previous day.

Every specimen showing an important discrepancy is retested using both methods.

Table 42 presents an outline of a representative day's tests taken from the laboratory records of June 26, 1924.

TABLE 42
One day's specimens with Wassermann test and author's precipitation system

PROCEDURES		AUTHOR'S PRECIPITATION SYSTEM				WASSER-MANN (SPINAL FLUID)							
		Routine test with serum		Presumptive procedure	Quantitative procedure	Spinal fluid procedure							
Results with all procedures													
Group number	Num-ber of sera in group	Cholesteri- nized antigen method	Serum:antigen dilution			Average findings (final result)	Serum:anti- gen dilution (special antigen) 15:1	Num- ber of sera	Maximum dilution of sera giving complete reaction	React- ing units	Num- ber of fluids	Serum:anti- gen dilution 15:1	Cholesteri- nized antigen method
			3:1	6:1	12:1								
1	141	—	—	—	—	—	Negative	4	1:1	4	7	—	—
2	30	++++	++++	++++	+++	+++	Positive	2	1:5	20	4	+++	++++
3	1	++++	++++	++++	+++	+++	Positive	1	1:10	40	1	+++	++++
4	2	++++	++++	++++	+++	+++	Positive	1	1:20	80	1	+++	++++
5	2	++++	++++	++++	+++	+++	Positive	1	1:30	120	1	+++	++++
6	2	++++	++++	++++	+++	+++	Positive	1	1:110	440	1	+++	++++
7	1	++++	—	—	+	+	Positive	1			1	++	+
8	2	++++	++++	++++	+++	+++	Positive	1			1	++	+
9	1	++++	—	—	+	+	Positive	1			1	±	—
10	2	++	++++	++++	+++	+++	Positive	1			1	—	++++
11	1	++	+	++++	+++	+++	Positive	1			1	—	++++
													Ac.*

CHAPTER XXIII

THE ROUTINE TEST IN THE DIAGNOSIS OF SYPHILIS

A series of studies on the clinical value of the routine test was undertaken by the author and Dr. Harther L. Keim of the Department of Dermatology and Syphilology of the University of Michigan Hospital. These studies are being continued and some have already been published in detail elsewhere (59). In this chapter, a resumé of the results based on 3600 examinations is presented.

Plan of investigation. Blood specimens were obtained from patients entering the University of Michigan Hospital and examined with the author's test and 2 separate Wassermann tests. Each specimen was divided into two parts, one being submitted to the laboratory of the Michigan Psychopathic Hospital for a Wassermann test, and the other to this laboratory for a Wassermann and a Kahn test. The specimens submitted bore no indication as to the clinical diagnosis. In accordance with the routine procedure with all blood specimens received at the Lansing laboratory, the Kahn tests were carried out on the same day the specimens arrived, and the Wassermann tests on the following day. The Wassermann test was performed with a cholesterinized antigen, using 1 hour fixation at icebox temperature, while at the Ann Arbor laboratory a cholesterinized antigen with overnight fixation at icebox temperature was employed. The author's test was thus compared with two different Wassermann systems—one believed to be highly sensitive, and the other more conservative in character. The results of all examinations were submitted to Dr. Keim who compared them with the clinical diagnoses.

The results with the author's test as recorded in the following tables represent the average findings of the 3 tubes. Thus, if the reaction is recorded as four plus, the

TABLE 43
Classification of cases studied

TYPE OF SYPHILIS	NUMBER OF CASES
Primary.....	47
Secondary.....	75
Tertiary—excluding cerebrospinal.....	130
Cerebrospinal.....	274
Latent.....	340
Congenital.....	65
Non-syphilitic controls.....	2,675
Total.....	3,606

TABLE 44
Primary syphilis

NUMBER OF CASES	REACTIONS WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION	
		18 hours	1 hour
Agreement			
21	++++	++++	++++
17	—	—	—
Divergence			
1	++++	++++	+++
1	++++	++++	++
2	++++	++++	+
1	++++	++	++++
1	++++	—	++++
1	++	++++	—
1	±	—	—
1	+	—	—

precipitation results in each of the 3 tubes was four plus; if two plus, the results in the three tubes might have been, for example, negative, two plus, four plus.

Primary syphilis. An examination of table 44 indicates that in primary syphilis the author's test is somewhat more sensitive than either of the two Wassermann tests.

Secondary syphilis. In secondary syphilis there appears to be general agreement with all methods (table 45).

Tertiary syphilis. In tertiary syphilis excluding cerebrospinal (table 46) the author's test appears to be considerably more sensitive than either of the two Wassermann tests. This is particularly illustrated in syphilis of cutaneous and mucous membrane involvement as well as of the osseous

TABLE 45
Secondary syphilis

NUMBER OF CASES	REACTIONS WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		TREATMENT
		18 hours	1 hour	
Agreement				
70	++++	++++	++++	
Divergence				
1	++++	++++	++	No history
1	++++	++	++++	No history
1	++	++++	+	No history
1	+	±	±	Indifferent
1	-	±	-	Well

system. In syphilis of the visceral system the precipitation test compares in sensitiveness with that of the two Wassermann tests.

Cerebrospinal syphilis. In cerebrospinal syphilis of a diffuse type as well as in general paresis (table 47) the author's test is comparable in sensitiveness with the prolonged fixation Wassermann test. In tabes dorsalis this Wassermann test appears to be more sensitive.

Latent syphilis. In latent syphilis (table 48) the sensitiveness of the author's test compares favorably with the prolonged fixation Wassermann test.

TABLE 46
Tertiary syphilis excluding cerebrospinal

TYPE OF INVOLVEMENT	NUM- BER OF CASES	REACTIONS WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		TREATMENT
			18 hours	1 hour	
Agreement					
Cutaneous and mucous membrane.....	40	++++	++++	++++	
	5	—	—	—	
Osseous system.....	17	++++	++++	++++	
	6	—	—	—	
Visceral system.....	27	++++	++++	++++	
	4	—	—	—	
Divergence					
Cutaneous and mucous membrane.....	1	++++	±	±	
	1	++++	—	++++	
	1	++	—	—	
	1	—	++	—	
Osseous system.....	1	++++	++++	+	
	1	++++	++++	±	
	1	++++	+++	±	
	1	++++	±	—	
	1	++++	—	++++	
	1	++++	—	—	
	1	+++	++++	++	
	1	++	+	—	
Visceral system.....	5	++++	++++	++	Indifferent
	2	++++	++++	+	Indifferent
	1	++++	++++	±	Indifferent
	1	+++	++++	++++	No history
	1	+++	++++	±	Indifferent
	1	+++	—	±	Thorough
	1	++	++++	++++	No history
	1	++	++++	+++	Indifferent
	1	++	++++	+++	Thorough
	1	++	—	—	Thorough
	1	+	++++	++	Indifferent
	1	+	—	—	No history
Central choroiditis and neuro-retinitis.....	1	++++	±	++++	No history
	1	++	—	—	No history

TABLE 47

Cerebrospinal syphilis

TYPE OF INVOLVEMENT	NUMBER OF CASES	REACTION WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		TREATMENT
			18 hours	1 hour	
Agreement					
Diffuse.....	66	++++	++++	++++	
	17	—	—	—	
Tabes dorsalis.....	33	++++	++++	++++	
	10	—	—	—	
General paresis.....	52	++++	++++	++++	
	3	—	—	—	
Divergence					
Diffuse.....	9	++++	++++	++	Indifferent Thorough Indifferent Indifferent Thorough Thorough Thorough No history Thorough Thorough Thorough No history Indifferent Indifferent Thorough Indifferent Thorough Indifferent No history
	13	++++	++++	+	
	2	++++	++	++	
	1	++++	—	+	
	1	++++	—	+	
	1	++++	—	—	
	3	++++	++	±	
	2	+++	+++	++	
	2	+++	++++	±	
	1	+++	—	—	
	1	+++	±	—	
	2	++	++	+	
	3	++	++++	++	
	1	++	++++	++++	
	1	++	—	±	
	1	+	++++	±	
	1	+	++++	+	
	1	±	++++	++	
	1	±	—	+	
	2	—	++++	—	
	2	—	++++	—	
	2	—	+++	—	
	1	—	++	—	

TABLE 47—Continued

TYPE OF INVOLVEMENT	NUMBER OF CASES	REACTION WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		TREATMENT
			18 hours	1 hour	
Divergence—Continued					
Tabes dorsalis.....	4	++++	++++	++	Thorough
	1	++++	++++	±	
	2	++++	++++	+	
	1	++++	++++	—	
	1	+++	++++	+	Thorough
	1	+++	++	+++	Indifferent
	1	+++	++++	++++	Thorough
	1	++	++++	+	No history
	1	+	++++	+	Indifferent
	1	+	++++	++++	No history
	1	±	++++	±	Indifferent
	1	—	++	—	Thorough
	1	—	++++	+	Indifferent
	1	—	++++	—	Thorough
General paresis.....	1	++++	++++	+++	Thorough
	2	++++	++++	+++	No history
	2	++++	++++	++	Indifferent
	3	++++	++++	±	Indifferent
	1	++++	+	++++	
	1	++++	++++	—	No history
	1	+++	++++	++++	No history
	1	+++	+++	+	Thorough
	1	++	++++	+	Thorough
	1	++	++	±	Indifferent
	1	++	+++	—	Thorough
	1	++	—	+	Thorough
	1	+	+	—	Indifferent
	1	+	++	—	Thorough
	1	—	++++	—	Indifferent
	1	—	++++	—	Thorough
1	—	++	—	Thorough	

Congenital syphilis. In congenital syphilis (table 49) the sensitiveness of the author's test is also apparently similar to that of the more sensitive Wassermann test.

TABLE 48
Latent syphilis

NUMBER OF CASES	REACTION WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		TREATMENT
		18 hours	1 hour	
Agreement				
166 51	++++ —	++++ —	++++ —	
Divergence				
5	++++	++++	+++	No history
15	++++	++++	++	No history
18	++++	++++	+	No history
6	++++	++++	±	No history
3	++++	++++	—	No history
1	++++	+++	—	Thorough
1	++++	++	—	No history
1	++++	—	++++	Thorough
2	++++	—	+	Thorough
1	++++	±	±	Thorough
3	++++	—	—	No history
1	+++	++++	++	No history
4	+++	++++	+	
1	+++	++	++	Indifferent
2	+++	+++	++	No history
1	+++	++++	—	No history
10	+++	++++	+	
1	+++	±	—	Thorough
1	+++	±	±	No history
1	+++	±	±	Indifferent
2	+++	—	—	Thorough
1	+++	—	+++	Thorough
4	++	++++	++	
9	++	++++	+	
2	++	+++	+++	Thorough
2	++	++++	—	Thorough
3	++	+	—	Thorough
2	++	±	±	Indifferent
3	++	—	—	Thorough
1	++	±	—	Thorough
1	+	++++	—	Thorough
1	+	++++	—	No history
1	+	—	±	Indifferent
1	+	—	—	Thorough
1	±	—	—	Thorough
1	±	++	—	Thorough
2	—	++	—	Thorough
6	—	++++	—	No history
1	—	++++	—	Thorough
1	—	++++	++	No history

Non-syphilitic controls. Of 2675 non-syphilitic controls (table 50) 2664 were negative with all methods. The 8 precipitation reactions given with the author's test are listed, giving also the date of examination in each case. This is done because the series of 3600 examinations contains

TABLE 49
Congenital syphilis

NUMBER OF CASES	REACTIONS WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		TREATMENT
		18 hours	1 hour	
Agreement				
36	++++	++++	++++	
9	—	—	—	
Divergence				
2	++++	++++	++	No history
1	++++	++++	+	Thorough
1	++++	++++	—	Thorough
1	++++	++	++	No history
1	++++	+	++	Thorough
1	++++	—	++++	Thorough
2	+++	+++	++	Thorough
1	+++	+	—	Thorough
1	++	++++	+++	Thorough
1	++	±	±	Thorough
1	+	++++	++	Thorough
2	+	—	—	No history
1	+	±	±	Thorough
1	±	++++	±	Thorough
2	—	++++	—	Thorough
1	—	++++	—	No history

some which were made with the author's earlier precipitation procedure. Only 2 of the 8 apparently non-specific reactions were obtained in 1924. The others, therefore, were obtained either with the earlier procedure or before the present procedure was fully standardized.

With regard to the application of the routine test to fluids from syphilitic lesions, our experience has been limited—due to lack of clinical material. On several occasions, we obtained precipitation reactions with fluids aspirated from chancres. These fluids gave negative Wassermann reactions and strong or weak precipitation reactions. The fluids, furthermore, were diluted with 1 or 2 parts of salt solution.

TABLE 50
Non-syphilitic controls

NUMBER OF CASES	REACTION WITH AUTHOR'S TEST	WASSERMANN REACTION WITH CHOLESTERINIZED ANTIGEN ICEBOX FIXATION		DIAGNOSIS	DATE OF EXAMINA- TION
		18 hours	1 hour		
2,664	—	—	—		
2	+	—	—	Alopecia areata	5-8-23
1	+++	—	++	Pompholyx	5-13-23
1	++	—	+	Acne rosacea	5-18-23
1	+	±	±	Acne vulgaris	11-23-23
1	++	—	—	Verruca plantaris	11-25-23
1	+	±	—	Pityriasis rosea	2-16-24
1	++	—	—	Mycelial dermatitis	4-10-24
1	—	±	—	Pneumonia	
1	—	++++	—	Deviated nasal septum	
1	—	++++	—	Tonsillitis	

SUMMARY

A study of the clinical value of the routine test was carried out with 3606 cases. Of these, 931 were cases representing various stages of syphilis. The results indicate that this test is equal in sensitiveness to a Wassermann test in which a cholesterolized antigen and 18 hour fixation are employed. The results further indicate that the test possesses a high degree of reliability as a diagnostic agent in syphilis.

CHAPTER XXIV

THE ROUTINE TEST IN CASES UNDERGOING TREATMENT

A large number of specimens are received in this laboratory from cases undergoing antisymphilitic treatment. These include specimens from about 600 syphilitic cases in the Michigan state prisons. The results of the examinations of these specimens with the Wassermann and Kahn tests, as tabulated in the laboratory records, give a fair indication of the relative sensitiveness of the 2 tests in sera from treated cases. Table 51 summarizes the findings in 1000 specimens received from April 1 to August 1, 1924 in which a history of treatment was specified.

It is evident from this table that, while the author's test is somewhat more sensitive in treated cases than the Wassermann test as carried out in this laboratory, there are some cases in which the Wassermann test gives more sensitive reactions.

It should be mentioned in this connection that specimens from treated cases are responsible for practically all the discrepancies in results of the Wassermann and author's tests which occur from day to day in the routine work in this laboratory. The serologic condition of the blood from such cases appears to be extremely unstable. Thus, repeated examinations of the same serum containing a considerable number of reacting substances may readily vary from two plus to four plus. A serum containing a comparatively small number of reacting substances may vary from negative to as high as two plus. These variations are likely to occur more often in the Wassermann test due to the variables inherent in the hemolytic system. Although the precipitation test appears to possess a high degree

TABLE 51
Comparative findings of Wassermann and Kahn tests in 1000 treated cases

NUMBER OF SPECI- MENS	KAHN RESULTS		WASSER- MANN RESULTS	NUMBER OF SPECI- MENS	KAHN RESULTS		WASSER- MANN RESULTS
	Reading of 3 tubes				Reading of 3 tubes		
Agreement of results with Wassermann and Kahn							
290	++++	++++	++++	12	-	+	+
21	+	++++	+++	21	-	+	±
19	-	++++	++	273	-	-	-
Kahn results stronger							
38	+	++++	++++	24	++++	++++	++++
7	-	+++	+++	34	++++	++++	++++
1	-	++	++	27	++++	++++	++++
1	-	±	++	6	++++	++++	++++
0	-	-	++	3	++++	++++	++++
6	-	+++	+++	22	++++	++++	++++
6	-	++	+++	30	++++	++++	++++
2	-	±	+	10	++++	++++	++++
0	-	-	-	9	++++	++++	++++
13	-	+	++	22	++	++	++
3	-	±	+	15	++	++	++
3	-	-	-	9	++	++	++
7	-	±	+	24	+	+	+
1	-	-	-	17	-	+	+
6	-	-	-	16	-	±	±
Wassermann results stronger							
38	+	++++	++++	24	++++	++++	++++
7	-	+++	+++	34	++++	++++	++++
1	-	++	++	27	++++	++++	++++
1	-	±	++	6	++++	++++	++++
0	-	-	++	3	++++	++++	++++
6	-	+++	+++	22	++++	++++	++++
6	-	++	+++	30	++++	++++	++++
2	-	±	+	10	++++	++++	++++
0	-	-	-	9	++++	++++	++++
13	-	+	++	22	++	++	++
3	-	±	+	15	++	++	++
3	-	-	-	9	++	++	++
7	-	±	+	24	+	+	+
1	-	-	-	17	-	+	+
6	-	-	-	16	-	±	±
Kahn results stronger							
38	+	++++	++++	24	++++	++++	++++
7	-	+++	+++	34	++++	++++	++++
1	-	++	++	27	++++	++++	++++
1	-	±	++	6	++++	++++	++++
0	-	-	++	3	++++	++++	++++
6	-	+++	+++	22	++++	++++	++++
6	-	++	+++	30	++++	++++	++++
2	-	±	+	10	++++	++++	++++
0	-	-	-	9	++++	++++	++++
13	-	+	++	22	++	++	++
3	-	±	+	15	++	++	++
3	-	-	-	9	++	++	++
7	-	±	+	24	+	+	+
1	-	-	-	17	-	+	+
6	-	-	-	16	-	±	±
Specimens anticomplementary in Wassermann							
1	++++	++++	++++	1	-	-	-
				Ac.*			Ac.

* Ac. = anticomplementary.

of constancy, it is not uncommon to obtain variable results with a serum from a treated case. Such a serum may give $-$, $+$, $++$ on the first examination and $-$, $++$, $++++$ on examinations the following day. As a rule, however, the final results show comparatively little variation since they are based on the average finding of the three tubes.

Based on the fact that syphilitic sera containing a lesser number of reacting substances require a comparatively small amount of antigen for complete precipitation, it is to be expected that many sera from treated cases will give weak or negative precipitation reactions in the first tube of the routine test and possibly in the second and give stronger reactions in the third tube which contains the smallest amount of antigen. This is illustrated in table 51. Rarely, stronger precipitation reactions are obtained in the first tube and weak or negative reactions in the second and third tube. In the case of such reactions, the examinations should be repeated to eliminate possible errors in measurements of antigen dilution and serum. In over 50,000 examinations with the present procedure, we have observed only two or three reactions in which on repetition the first tube persisted in showing a definite precipitate and the remaining tubes, weak or entire freedom from precipitate. The Wassermann results in these cases were one or two plus. In several other cases where such anomalous reactions occurred, they have been due to technical errors and, on repetition, the results were typical—the reactions being stronger in the tubes containing the lesser amount of antigen.

Furthermore, weak reactions in which the results in the three tubes are the same, such as $++$, $++$, $++$; $+$, $+$, $+$; and $\pm$, $\pm$, $\pm$, are also atypical in character and the specimens in such cases should be reexamined. The serum controls of such reactions should be examined with particular care for the presence of foreign particles.

If these reactions are obtained on repetition, the average findings should be taken as the final result. Such reactions, however, should be considered atypical.

It would be of interest to extend the studies on the routine test in treated cases to include the effect on the test of each course of treatment in an individual case. Such studies are necessarily for the clinician. We have had the opportunity to observe only a limited number of cases. Table 52 gives the results with the test in one such case. Each specimen of blood was obtained just before the treatment indicated.

TABLE 52
The routine test in a case during treatment

TREATMENT NUMBER	DATE	REACTION WITH ROUTINE TEST		
2	10- 3-23	++++	++++	++++
3	10- 4-23	++++	++++	++++
4	11-21-23	++++	++++	++++
6	11-23-23	+	++	++++
7	3-12-24	+	+++	++++
8	3-13-24	+	++	++++
9	3-14-24	+	++	++++

SUMMARY

The routine test possesses a relatively higher degree of sensitiveness with sera from treated cases than the Wassermann test as carried out in this laboratory. The weaker precipitation reactions in treated cases are weak or negative in the first tube containing the larger amount of antigen and relatively stronger in the second and third tubes containing the lesser amounts of antigen.

CHAPTER XXV

THE QUANTITATIVE PROCEDURE IN CASES UNDERGOING TREATMENT

It is well known that a so-called four plus reaction is a qualitative rather than a quantitative indicator of the serologic condition of a patient. Such a reaction merely means that there is a sufficient number of reacting substances in the serum to give complete precipitation (or complement fixation) under a set number of conditions. It does not indicate the relative number of such reacting substances. The routine precipitation test, consisting of 3 proportions of serum and antigen dilution, is quantitative in a limited degree. It was believed that a purely quantitative procedure would supply an important need particularly in treated cases. This procedure, as already described (Chapters VI and XIX), is based on a unit system and enables one to determine the relative number of reacting substances in a given serum.

It is not suggested that the quantitative procedure be applied to all sera received by a laboratory for examination. The procedure is applicable only to those sera which give four plus reactions with the routine test and is recommended for all such specimens wherever facilities permit. Aside from its value as a check on all four plus reactions, the quantitative procedure gives to the clinician a record of the relative number of reacting units present in a given serum and this information should prove a valuable addition to the clinical history in a given case.

The quantitative procedure is especially applicable to cases undergoing antisyphilitic treatment. The serum in a given case may show the presence of, let us say, 200 reacting units before the institution of treatment, and 40

units, for example, after the completion of a given course or courses of treatment. The reactions with the routine test would be four plus on both examinations, while the reduction from 200 to 40 units would be a helpful guide to the clinician in the continuation of treatment.

TABLE 53
The quantitative procedure in treated cases

CASE NUMBER	TREATMENT NUMBER	DATE (1924)	MAXIMUM DILUTION OF SERUM GIVING COMPLETE PRECIPITATION	NUMBER REACTING UNITS
1	I	4-17	1:40	160
	II	4-18	1:50	200
	III			
	IV	5-16	1:1	4
	V	5-19	1:1	4
	VI	5-19	1:1	4
2	I			
	II	4-28	1:20	80
	III	4-28	1:30	120
	IV	5-22	1:5	20
	V	5-23	1:10	40
	VI	5-24	1:5	20
3	IV	4-30	1:10	40
	V	5-2	1:1	4
	VI	5-2	1:1	4
4	IV	4-30	1:5	20
	V	5-2	1:1	4
5	IV	5-19	1:20	80
	V	5-19	1:20	80
	VI	5-22	1:5	20

The quantitative procedure should also prove helpful to clinicians when attempting to determine the value of new methods of antiluetic treatment. Assuming that the serologic status of a given case is related to the clinical status, then, if one form of therapy tends to reduce the

number of reacting substances in serum at a more rapid rate than another form, it might indicate that the one therapeutic agent possibly possesses greater antiluetic potency than the other.

It is hoped that clinicians will study the practical value of the quantitative procedure. Our opportunity for this study has been limited. Several of the cases which we have had an opportunity to observe are summarized in table 53. The treatments in these cases were administered at the Dermatological Clinic of the University of Michigan Hospital and specimens of blood were examined before each treatment. Courses of three treatments each were administered with rest periods of some weeks between these courses. It will be noted that we have no complete record of treatment in a single case. The cases recorded, however, indicate reduction in the number of reacting units due to treatment. This is particularly indicated after the rest periods following the first course of treatments (cases 1 and 2).

SUMMARY

The quantitative procedure is an indicator of the relative number of syphilitic reacting substances in serum and is of particular value in cases undergoing treatment. It may also be of value in studying the comparative effects of different modes of treatment.

CHAPTER XXVI

APPLICATION OF SPINAL FLUID PROCEDURE

This chapter presents data on the diagnostic value of the author's spinal fluid procedure in neurosyphilis. This procedure is carried out in a routine manner with all spinal fluids sent to this laboratory for serologic examinations. Clinical studies are now in progress in conjunction with Dr. Keim. These studies will be published in separate papers.

To date, a total of 750 spinal fluid examinations have been made. A Wassermann test was also made in each case, using a cholesterinized antigen and a fixation period of 1 hour in the icebox. It was early observed that Wassermann tests carried out with a cholesterin-free antigen gave less sensitive reactions than the precipitation procedure. Wassermann tests with a cholesterinized antigen, however, gave results apparently comparable with the precipitation test.

Table 54 gives comparative findings of Wassermann test and author's procedure with 750 spinal fluids. Of this number, 394 were positive and 276 negative by both methods. Of 10 fluids which were anticomplementary in the Wassermann test, 9 were positive and 1 was negative with the precipitation procedure. Of the remaining 70 fluids showing disagreement in results, the Wassermann test shows a somewhat higher degree of sensitiveness, although a number of fluids from neurosyphilis gave positive results with the precipitation procedure and negative with the Wassermann.

As is true in the case of serum, practically all discrepancies in the results of the Wassermann and precipitation tests were obtained with fluids from patients undergoing anti-syphilitic therapy. The results with such fluids indicate

some interesting differences in their behavior towards the precipitation and complement fixation tests. In some cases the results are consistently positive with both methods after prolonged treatment; in others, the results are consistently positive with one and negative with the other procedure.

TABLE 54

Comparative findings of Wassermann and author's test with 750 spinal fluids

NUMBER OF SPECIMENS	WASSERMANN RESULTS	KAHN RESULTS
Results same with both tests		
394	++++	++++
276	—	—
Fluids anticomplementary in Wassermann		
9	Ac.*	++++
1	Ac.	—
Results stronger with Wassermann		
10	++++	++
16	++++	+
4	++++	—
1	+++	+
2	+++	—
3	++	±
8	±	—
Results stronger with author's test		
1	++	++++
6	+	++++
3	—	++++
3	+	+++
3	±	++
10	—	±

* Ac. = anticomplementary.

Regarding the specificity of the precipitation procedure, we have no record of so-called false reactions. All fluids in which the precipitation procedure was positive and the Wassermann negative came from cases undergoing anti-

sypilitic treatment. It appears that definite precipitation reactions with the spinal fluid procedure have the same reliability as definite reactions with the routine test with serum.

SUMMARY

Based on findings with 750 spinal fluids, the author's precipitation procedure appears to be a valuable aid in the diagnosis of neurosyphilis.

CHAPTER XXVII

APPLICATION OF PRESUMPTIVE PROCEDURE

It is frequently desired to eliminate the possibility of syphilitic infection in a given case. Indeed, because of the innumerable manifestations of syphilis, this elimination is often of great importance. Largely to fill this need in the serum diagnosis of syphilis, the presumptive procedure was evolved. This procedure should also be of value as a check on the routine tests in cases undergoing antisiphilitic treatment.

The principles upon which this procedure is based as well as the technical details are fully considered elsewhere in this volume (Chapters XIV and XXI). Briefly, the procedure is a qualitative method of high sensitiveness, the results being interpreted as positive or negative (and not in terms of plus signs) depending on the presence or absence of a definite precipitate.

The procedure is now employed in a routine manner in this laboratory and our experience with it is based on over 4000 examinations. Its value is, in our experience, considerable. All sera giving positive reactions with the routine test must necessarily give positive reactions with the presumptive procedure. Every positive reaction is thus checked with a procedure in which an antigen different from the standard is employed. In the case of sera giving doubtful reactions with the routine test, the presumptive procedure almost invariably gives positive reactions. In the few cases, where this procedure is negative, it is found that the doubtful reaction in the routine test is due to the presence of foreign particles in the serum. We thus have in this presumptive procedure a valuable check on doubtful reactions.

It should be indicated that the results with the presumptive procedure are not at present reported to physicians. If, for example, the routine test with a given serum is one plus and the presumptive procedure is positive, only one plus is reported. It is not intended that the presumptive procedure should supplant the routine test, which, as already pointed out, was evolved not with a view to rendering it of the greatest sensitiveness but rather of a degree of sensitiveness conforming with reliability as a diagnostic test. The presumptive procedure, due to its high sensitiveness, may, in isolated cases, give non-specific reactions and is therefore not intended to be employed as a diagnostic method. In the course of time we expect to report the results with this procedure to physicians with the notation as to its proper interpretation.

This brings up the question of the specificity of the presumptive procedure. Obviously this is a problem for the clinician. Only careful clinical studies can determine whether or not a positive reaction with this procedure against a negative reaction with the routine test is suggestive of syphilis. In so far as our routine examinations with this procedure indicate, we are dealing with a method which is practically free from false reactions. In a daily run of 200 examinations it is usually found that the weak reactions and possibly 1 or 2 negative reactions with the routine test are positive with the presumptive procedure, and in the case of the one or two negative reactions there is usually a history of treatment. This suggests that we are probably dealing with a method which is specific in the great majority of cases.

It is not to be assumed that the presumptive procedure is positive in all cases of treated syphilis, particularly those which have undergone intensive treatment. Many sera from treated cases reach this laboratory daily in which this procedure gives negative results. It is, however,

more sensitive than the routine test. Since the routine test is comparable in sensitiveness to a Wassermann test carried out with a cholesterinized antigen and overnight fixation at icebox temperature as indicated in Chapter XXIII, it would appear that we are dealing in the presumptive procedure with a method of unusually high sensitiveness.

It is believed that this procedure should give good results with fluids from syphilitic lesions, particularly the chancre.

TABLE 55
The presumptive procedure in 4374 examinations

NUMBER OF SPECIMENS	RESULTS				
	Presumptive procedure	Routine test			Average
		3 tube reading			
607	Positive	++++	++++	++++	++++
74	Positive	++	++++	++++	+++
106	Positive	—	++++	++++	+++
82	Positive	—	++	++++	++
72	Positive	—	+	++	+
48	Positive	—	±	+	±
18	Positive	—	—	—	—
3,367	Negative	—	—	—	—

While not intended as a general diagnostic agent, the presumptive procedure, when properly interpreted by the clinician, should prove helpful in the diagnosis of isolated cases. It is well known that there are certain cases, especially of early primary, latent, hereditary and neurosyphilis in which no serologic test, no matter how sensitive, detects the syphilitic reacting substances. Indeed, as indicated in the series of tables in Chapter XXIII, there is a large number of cases of syphilis of all stages except secondary in which all the serologic reactions are negative. It is clear, therefore, that a test possessing a higher degree of sensitiveness than the routine test should prove of diagnostic value in special cases. On the other hand,

the importance of correct diagnosis of syphilis is such, particularly in view of the stigma associated with this disease, that it is essential to employ the utmost care in the use of the presumptive procedure as a diagnostic agent. Our basic reason for naming it "presumptive procedure" was to prevent its general use in diagnosis.

SUMMARY

The presumptive procedure, due to its high sensitiveness, is of especial value when it is desired to eliminate the possibility of syphilitic infection in a given case. The procedure is of value also as a serologic check in treated cases. It may have further application, when properly interpreted, in the diagnosis of obscure manifestations of syphilis.

PART VI
GENERAL CONSIDERATIONS

CHAPTER XXVIII

SUMMARY OF AUTHOR'S STUDIES—WITH CONSIDERATIONS OF SOME FURTHER INVESTIGATIONS

Although there exists an extensive literature on the serum diagnosis of syphilis, many of the fundamental problems involved are still unsolved. We still know little of the relation of heart muscle antigen to syphilis, neither do we know the basic physico-chemical laws which govern this relation, nor the true clinical interpretation of serologic reactions. This comparative lack of knowledge is, in our opinion, largely explained by the numerous and well known difficulties inherent in the phenomenon of complement fixation with heart muscle extract as antigen. A massive amount of experimental work had to be done in overcoming these difficulties in order to evolve a dependable complement fixation test for syphilis. Workers have thus been giving their attention to practical phases and as a result we have a large number of tests but no solution of the fundamental problems involved.

A practical precipitation test, in our opinion, offers a possible medium for the solution of many of these problems. Instead of judging the interreaction between serum and antigen by the disappearance of complement, and the disappearance of complement, in turn, by the hemolysis of red cells—as is done in complement fixation—the interreaction between serum and antigen is judged directly by the presence or absence of a precipitate. Added to this there is the probability that the elimination of incubation, which is accomplished in the procedures embraced by the author's precipitation system, is likely to eliminate many non-specific factors accompanying the incubation of biologic

fluids. It is clear therefore that these procedures should prove especially adapted to research investigations.

The studies presented in this volume have been limited to investigations of some of the principles governing the phenomenon of precipitation in syphilis with special reference to the evolvement of a practical precipitation test. No attempt has been made to solve the fundamental problems to which we have just referred. We have attempted rather to bring the test to such a degree of perfection as to assure dependability as a diagnostic method. The test could not be employed as a medium in research until its practical application was assured. An attempt is made in this chapter to briefly review the problems which we have already investigated and to indicate some further problems which suggested themselves during the course of our studies.

CONCENTRATION OF INGREDIENTS

Our investigations have shown that one of the first principles governing the precipitation phenomenon in syphilis is concentration of the ingredients. Antigen, serum and physiologic salt solution must be so employed as to assure a high degree of concentration. The mere concentration of antigen, however, is apparently not the final criterion in antigen preparation. A direct alcoholic extract of powdered heart muscle is obviously of high concentration, yet it does not compare in sensitiveness with an extract of the same heart muscle from which a given amount of ether soluble elements has been previously removed. Neither does a direct alcoholic extract compare in sensitiveness with a Noguchi or acetone insoluble extract or one prepared according to Kolmer.

Unpublished experiments have further shown that an acetone insoluble antigen of high lipid concentration is apparently less sensitive than one of lesser lipid concentra-

tion. This is illustrated by the following experiment: Two 25 gram quantities of powdered beef heart were each extracted in 125 cc. of 96 per cent alcohol for 3 days at 21°C. The extracts were each evaporated to dryness and the individual residues dissolved in small amounts of ether. An excess of acetone was added to each of the ether extracts and the acetone insoluble residues dissolved in 125 cc. absolute alcohol in one case and 62.5 cc. absolute alcohol in the other. After cholesterinizing these 2 acetone insoluble extracts by adding 6 mgm. of cholesterin per cubic centimeter and testing them with syphilitic serum, it was found that the less concentrated extract, prepared by dissolving the acetone insoluble residue in 125 cc. alcohol, gave more sensitive reactions than the more concentrated extract. This experiment indicates that concentration of beef heart lipoids may, under certain conditions, interfere with precipitation in syphilis.

Another experiment giving the same indication has already been discussed in the text. If to our standard antigen are added some acetone insoluble lipoids obtained from the ether extract and a secondary alcoholic extract, the final product, although obviously richer in beef heart lipoids than the standard antigen, is nevertheless less sensitive with syphilitic sera than the standard antigen.

These experiments indicate that the principle of concentration has its limitations. While the nature of the lipoids present in an antigen is undoubtedly of first importance, it is not unlikely that too high a degree of concentration even of specific lipoids may inhibit precipitation. It is obvious, therefore, that many problems related to concentration of lipoids still require solution.

The recent employment by different workers (Meinicke, Dujarric de la Riviere and Gallerand) of resinous matter in connection with antigen preparation may be related to concentration. We early observed that when

our antigen was in contact with rubber stoppers for any length of time the behavior of the final product with serum was considerably modified. This product produced far heavier precipitates with syphilitic sera than the standard antigen, and also showed a tendency for weak precipitation with non-syphilitic sera. We are inclined to think that many lipoid-like or resinous substances are capable of increasing the sensitiveness of a given extract and that this increase in sensitiveness is due in part to the increase in final concentration. It is not unlikely that the increase in concentration is one of the important reasons for the increased sensitiveness of a given antigen after the addition of cholesterin.

Our experiments in connection with the delaying effect of salt solution on precipitation, although relatively complete in character, could nevertheless be profitably extended. Careful quantitative studies on the effect of dilution on precipitation in syphilis may not only throw further light on this problem, but may help solve some of the problems associated with precipitation and agglutination of bacterial antigens. It may be of interest to note that preliminary experiments by Miss Kendrick and the author on the effect of dilution on agglutination of *B. typhosus* by anti-typhoid serum failed to show an effect in any way comparable to the effect of dilution in precipitation with antigen and syphilitic serum.

Of interest in connection with the dilution of antigen with physiologic salt solution would be studies on the use of various concentrations of the salt solution as well as on the use of different inorganic salts, such as potassium chloride and sodium sulphate and some organic salts, such as sodium benzoate and sodium oxalate. Higher concentrations of salt solution have been utilized by Meinicke and by Bruck in their respective methods. The determination of the extent to which the hastening effect of salt concentration

on precipitation would neutralize the delaying effect of dilution would make an interesting contribution. The use of salts other than sodium chloride has been studied in connection with other precipitation tests and such a study should be of interest in connection with this test.

Another problem worthy of further study is the effect of dilution on precipitates already formed. We have shown that dilution tends to break up precipitates in serum-antigen mixtures and if sufficiently high may actually render them invisible. It would be of value to determine whether these precipitates go back into solution or whether it is merely the cohesive power of the particles that is affected by the dilution.

UNSTABLE ANTIGEN DILUTION

After the importance of concentration in the precipitation phenomenon in syphilis had been fully established, observations indicated that a cholesterinized alcoholic extract prepared from dried heart muscle from which the ether extractives had been previously removed (Neumann and Gager) would meet the requirements of suitable concentration for a precipitation test. Further observations showed that the use of this antigen in an undiluted form, while giving precipitates with syphilitic sera, gave non-specific reactions. It seemed that dilution of the antigen with salt solution was desirable in a precipitation test, provided a minimum amount of salt solution was employed. Experiments showed that approximately 3 parts of salt solution represented the minimum amount which, when added to one part of antigen, produced an opalescent mixture. This mixture gave immediate precipitation reactions with highly potent syphilitic sera, but with sera of less potency, precipitates resulted only after overnight incubation. It was observed in connection with these antigen-saline mixtures that some remained opalescent

for many hours, while others precipitated spontaneously within a short period, indicating a high degree of instability with reference to precipitation. It was further observed that such unstable antigen mixtures gave more sensitive precipitation reactions with syphilitic sera than the more stable antigen mixtures. This led to the view that by making use of instability as well as of concentration an antigen mixture might be rendered sufficiently sensitive to give immediate precipitation reactions—not only with highly potent sera but also with sera of weak potency.

Observations on the behavior of the antigen with various amounts of salt solution soon showed that (a) 1 cc. amounts diluted with 0.5 cc. saline resulted in a mixture containing a precipitate which was stable and insoluble on further addition of salt solution; (b) 1 cc. antigen diluted with 1, 1.5 or 2 cc. salt solution, resulted in mixtures containing precipitates which were readily soluble on further addition of salt solution and apparently, therefore, unstable with reference to precipitation. When each of these mixtures was employed with different sera, it was observed that the antigen precipitates dissolved immediately. In the case of syphilitic sera, however, a new precipitate appeared within a minute or two, particularly after some agitation. As might have been expected, of the 1 + 1, 1 + 1.5 and 1 + 2 proportions of antigen and salt solution tested, 1 + 1, the one containing the least amount of salt solution, gave the most sensitive reactions with syphilitic sera and formed the basis for further study.

Since for a highly sensitive antigen-salt solution mixture it was essential that the amount of salt solution be reduced to a minimum, it seemed possible that concentration rather than the presence of an unstable precipitate was responsible for high sensitiveness. Fortunately this question lent itself readily to experimental study. In one case, syphilitic serum was diluted with a very small amount of salt solution

before adding the serum to the antigen dilution. In a similar case, the same amount of salt solution was added to the antigen dilution—causing solution of the antigen precipitate—before mixing it with undiluted serum. It was observed that there was a marked difference in the sensitiveness of the final precipitation reactions in the two cases. The addition of the salt solution to the serum caused practically no reduction in sensitiveness, due undoubtedly to the fact that the amount of salt solution added was so small that the reduction in the degree of concentration was negligible. The addition of the same amount of salt solution to the antigen dilution, however, resulted in a marked reduction in sensitiveness of the final precipitation reactions compared with the controls. The explanation for the reduction in sensitiveness undoubtedly lies in the physical change of the antigen-saline precipitate. This precipitate was dissolved by the small amount of salt solution. We were no longer dealing in the antigen dilution with a precipitate of high instability but with one which was comparatively stable and therefore less sensitive with sera.

Our studies in connection with the ingredients of the antigen dilution which are responsible for specific precipitation also appear to prove our contention that the physical condition of the antigen-saline precipitate is largely responsible for the high sensitiveness of the antigen dilution. If the standard antigen dilution is centrifuged and the precipitate is separated from the supernatant fluid and suspended in a solution consisting of equal parts of 95 per cent alcohol and salt solution—similar to the alcohol concentration of the supernatant fluid—the final mixture is practically as sensitive as the original antigen dilution. If, however, the antigen dilution precipitate is suspended in a solution consisting of equal parts of distilled water and saline, the final antigen mixture is considerably less

sensitive than the standard antigen dilution. The probable explanation for this is that, when the antigen dilution precipitate is suspended in the alcohol-saline solution, the physical character of the precipitate is not changed, while, when suspended in the distilled water-saline solution, its character is changed, the precipitated particles being very fine instead of heavy as in the antigen dilution.

As further evidence of the high instability of the antigen dilution we might refer to our experiments on the effect of age on this dilution. It was observed that after diluting antigen with salt solution the mixture increased in sensitiveness on standing. After a somewhat prolonged period, the mixture became incapable of holding the cholesterin in solution. The probable explanation for the increased sensitiveness is that the antigen dilution becomes more unstable on standing. Soon the degree of instability reaches a stage when the cholesterin, due to its weak union with the other lipoids, begins to crystallize out.

The relation between the antigen-saline precipitate and the serum-antigen precipitate may be summarized as follows:

1. Antigen + salt solution = antigen dilution containing a precipitate.

2. Antigen dilution + syphilitic serum = solution of antigen precipitate followed by immediate formation of new precipitate.

3. Antigen dilution + nonsyphilitic serum = solution of antigen precipitate with no formation of new precipitate.

In the case of positive serum, therefore, we have within a few minutes the following changes:

Precipitate $\longrightarrow$ solution $\longrightarrow$ new precipitate

It is clear that careful investigations of each of these conditions are essential if we are to gain a fuller understanding of the mechanism of the test.

Although some chemical studies on the antigen and serum precipitates are recorded in the literature in connec-

tion with other precipitation tests, it appears to us that our test is particularly adapted for more extensive studies of this kind. We have shown that the precipitate in the antigen dilution and not the supernatant fluid possesses the antigenic properties. Under these conditions, chemical studies of the antigen precipitate parallel with such studies on the final precipitate in positive serum should offer a fruitful field of investigation. It may be of interest to note in this connection that, while the precipitate formed in serum is readily soluble in weak alkali, such as $N/50$ NaOH, the antigen precipitate is insoluble in normal alkali, indicating a marked difference in the reactions of these two precipitates to alkali.

The many problems associated with instability of antigen dilution and the behavior of this dilution with serum belong to the colloidal chemist. When diluting antigen with salt solution there is a physical rather than a chemical change, resulting in a suspension of lipoid particles. This was early recognized by Sachs and other pioneer students in this field. We have reason to believe further that the electrolytic action of the salt solution probably plays but a small rôle. We have observed that antigen diluted with distilled water results in mixtures readily capable of producing precipitates with syphilitic sera. The greater sensitiveness of our antigen dilution compared with antigen dilutions employed in other precipitation tests undoubtedly lies in the physical character of the lipoid particles. The difficulty of studying colloidal problems in connection with an alcoholic solution of lipoids is fully recognized. It is hoped, however, that the simplicity of our test will render such studies possible.

RELATION BETWEEN SERUM AND ANTIGEN

Although the quantitative relation between serum and antigen has been studied in connection with other serologic

tests for syphilis, none of these studies, to our knowledge, led to the definite character of the findings made possible by the author's precipitation method. We have shown, for example, that a small excess of antigenic substances in relation to the syphilitic reacting substances in the serum readily inhibits precipitation. In other words, the number of antigen elements must approach the number of serum substances for complete precipitation. But this does not hold true when the serum substances are in excess of the antigen elements. A strongly positive serum reacts readily, with a large as well as a small amount of antigen. The explanation for this will ultimately be found by the physical chemist when the mechanism of the precipitation reaction as a whole is explained.

An interesting problem and one readily subject to quantitative measurements is, in our opinion, that dealing with the absorption of serum reacting substances with antigen. Our preliminary experiments have shown that the serum substances may be readily removed with antigen in somewhat the same manner as typhoid agglutinins, for example, are absorbed by suspensions of *B. typhosus*. It should be of value to carry out such studies on a larger scale. It is conceivable that, after absorption of the reacting substances of serum by antigen, some means may be found of disrupting the union between antigen and serum substances. This might make possible a study of the nature of the serum substances responsible for specific precipitation in syphilis.

SENSITIVENESS AND UNIFORMITY OF ANTIGEN

It is not frequently recognized that all alcoholic extracts of heart muscle, no matter how prepared, are capable of producing precipitates with syphilitic sera under proper conditions—and even under adverse conditions, if the sera are very potent, such as from cases of secondary syphilis. It has long been known that practically any kind of extract

antigen will "fix" complement under proper conditions, which explains why there are so many complement fixation tests. The interest in precipitation tests in recent years has naturally brought forth and will undoubtedly continue to bring forth new tests. It is clear, however, that this alone will not solve the numerous problems associated with the serum diagnosis in syphilis and that it is of first importance to gain a more complete understanding of the principles underlying precipitation.

After embodying the knowledge gained from our studies on concentration, instability of antigen dilution and quantitative relation between serum and antigen in the construction of a precipitation test, we believed for a time that the major problems connected with such a test had been solved. It soon became clear that a better understanding of the factors governing the sensitiveness and uniformity of antigen had to be gained if our test were to possess practical value. We had not recognized the full importance of this previously because we had been accustomed to make large amounts of antigen at a time and we had no opportunity of observing the fluctuations in sensitiveness which we should have observed, had we prepared new lots of antigen at frequent intervals.

It is of interest to note that practically all of the precipitation tests published have been subsequently modified to a considerable degree by their authors. The reason for this, in our opinion, lies chiefly in fluctuations in sensitiveness of antigen. Such fluctuations probably occur in a greater degree in the case of antigens prepared from wet heart muscle because of their relatively small content of specific lipoids. The marked difference in results in the hands of different workers with the Sachs-Georgi reaction, for example, is undoubtedly due largely to fluctuations in the amount of specific lipoids of the antigens. Indeed, some workers have found it convenient to prepare several

lots of Sachs-Georgi antigen at a time, with the hope that one of the lots would possess a desirable degree of sensitiveness. The fluctuations in sensitiveness of the antigen used during the development of our test led us to undertake a study of the factors which govern both sensitiveness and uniformity of antigen.

Preliminary experiments indicated that small variations in the alcohol extraction method employed in antigen preparation, had little effect on antigen sensitiveness, while small variations in the ether extraction method had a marked effect on antigen sensitiveness. Further experiments soon established that there was a direct relationship between the amount of ether extractives a given antigen contained and the sensitiveness of the antigen. It was found possible to prepare antigens of high or low sensitiveness by quantitatively increasing or decreasing the amount of ether extractives. This led to the development of a new procedure for antigen preparation, a procedure in which the factors responsible for variation in antigen sensitiveness were largely eliminated.

Having experimentally established the conditions governing antigen sensitiveness, it was relatively simple to produce an antigen of higher sensitiveness than the standard for use in the presumptive procedure. An antigen of still higher sensitiveness can be produced, but such an antigen would be likely to lack the high degree of practicability in its use with serum possessed by the antigen chosen for this procedure.

Further studies on antigen sensitiveness in relation to ether extractives should be, in our opinion, of especial interest. The ether extractives of beef heart are markedly insoluble in salt solution, no matter how large an excess of the latter is employed. The alcohol extractives of beef heart, from which the ether extractives have been removed, possess a high degree of solubility in salt solution, dissolving

readily in a small excess of the latter. If an ether extract of beef heart is evaporated to dryness and the residue shaken up in alcohol, only a small portion will dissolve in the alcohol. But this alcoholic solution of ether-soluble lipoids when mixed with an excess of salt solution contains a precipitate insoluble in salt solution. Yet, the alcoholic extract can hold in solution a considerable portion of ether extractives and the precipitate formed on mixing this extract with salt solution is readily soluble in an excess of salt solution. Since lipoids are ordinarily soluble in other lipoids, it is possible that the ether extractives are so modified by their union with alcohol extractives as to become, up to a certain degree, soluble in salt solution. If the ether extractives are excessive in relation to the alcohol extractives, this solubility power is lost. This may explain the fact that, when adding increasing amounts of ether extractives to alcohol extract and subsequently mixing the final extract with salt solution, there is observed gradual gradations from opalescence or apparent solubility of the lipoids to turbidity or relative solubility to final precipitation or total insolubility of the lipoids. It thus seems possible that the ether extractives may possess little or no antigenic powers in themselves and their ability to increase the sensitiveness of antigen may be due to the physical effect they exert on the specific alcohol extractives by decreasing their solubility.

It should be pointed out that the governing principles of precipitation in syphilis considered in this volume are not limited in their application to our standard antigen but apply to other antigens as well. Any alcoholic extract from which the ether or acetone-soluble lipoids have been removed may give reasonably good results when employing the technic of our procedures. Of interest is our finding that an antigen prepared according to Kolmer and one prepared according to Noguchi—when containing 0.6

per cent cholesterolin—approach the sensitiveness of our standard antigen. Certain practical considerations, however, as described under our special study in connection with the use of different antigens (Chapter XIII) preclude their use in our procedures.

We have not had occasion to prepare antigen from sources other than beef heart. Whether or not antigens prepared from other animal hearts or other tissues would prove practicable should make an interesting study.

It is generally believed that, after beef heart has been extracted with ether and subsequently extracted with alcohol, a secondary alcoholic extract contains antigenic lipoids not present in the first alcoholic extract (Erlandson, Neumann and Gager, Kolmer). On this basis it seemed possible that an antigen superior to our standard might be prepared if we extracted the beef heart 2 or 3 times with alcohol and combined these extracts. However, our experience with such antigens as compared with the standard antigen, has not convinced us of their superiority. We observed that, if after the first alcoholic extraction of beef heart and filtration of the extract, pressure was applied to the beef heart residue and the adhering extract removed as completely as possible, a secondary alcoholic extract of the beef heart residue was practically free from lipoids. This seems to indicate that there is probably little antigenic value in the secondary alcoholic extraction, if the first extract is completely removed.

The most important problem in connection with antigen studies is careful quantitative chemical analysis of the ether and alcohol extracts in relation to their antigenic properties in the precipitation test. Such chemical studies in connection with the complement fixation test have had many drawbacks, particularly because of the inherent anticomplementary properties of many extract lipoids. These difficulties will not be encountered in our test and

such studies should give valuable information. Indeed, chemical studies of this character are essential if we are to learn the true relation of beef heart antigen to syphilis.

STUDIES ON SERUM

Heating experiments on serum in connection with our precipitation test indicated a relatively high degree of thermostability of the syphilitic reacting substances. It is generally accepted in connection with the complement fixation test that these substances are thermolabile. Many Wassermann tests have been evolved with unheated sera by different workers, largely to overcome the apparent destruction of these substances after heating the sera at 56°C. We, ourselves, at one time differentiated the reacting elements of syphilitic serum from specific antibodies by the relative thermostability of the latter and thermolability of the former. It is probable, as suggested in the text, that the apparent destruction of reacting substances in syphilitic serum observed by different workers in connection with complement fixation was due to factors unrelated to serum, such as the kind of antigen used in the test and the length of the fixation period.

Why it should be essential to inactivate serum for our test is a problem well worthy of investigation. We have observed that even 5 minutes' heating at 56°C. is sufficient to render many sera positive. Contrary to the general belief that the substances in weak sera are particularly destructible at 56°C., our experiments have shown that the weaker the serum the more prolonged is the heating at 56°C. required to bring the reaction to apparently full strength. After 1 to 2 hours' heating at 56°C., weaker sera frequently show stronger reactions than after 30 minutes' heating.

These findings have been recently corroborated by Miss Kendrick in a more extended series of heating experiments. Should they be further corroborated by other investigators,

it might be well to extend the heating period of sera in our test to 1 hour or even longer. Regarding the effect on reacting substances of a temperature higher than 56°C., our findings indicate that heating at 62°C. causes comparatively little destruction of these substances until extended to one or more hours.

Experiments in connection with the effect of age and bacterial contamination of sera on the author's test showed that there was relatively little effect compared with that on the Wassermann test. When this finding was discussed with Dr. Novy, he suggested that it would be of interest to inoculate serum with organisms having proteolytic powers and then study the effect on our test. Such experiments could be extended further by the digestion of sera with proteolytic ferments.

Studies which we have carried out in a preliminary manner and which are worth extending are those related to the effect of adding acid or alkali to serum. Negative sera to which acid, in sufficient concentration, has been added give precipitation reactions. Positive sera, on the other hand, to which alkali has been added give negative reactions. Of particular interest is the apparent zone phenomenon observed in the acid experiments. This phenomenon may be related to the zone phenomena observed in connection with acid precipitation or agglutination in other fields.

The all important problem in connection with serum is the determination of the nature of the substance or substances responsible for precipitation with antigen. Unpublished experiments on the concentration of globulins in serum with ammonium sulphate as suggested by Herrold and subsequent employment of the concentrated globulin solutions with standard antigen dilution did not give promising results. Many concentrated globulin solutions from sera of nonsyphilitic cases gave precipitates. It is

questionable whether the globulins alone are the carriers of the specific reacting substances in syphilitic serum. Chemical fractioning of serum and a study of each fraction with the precipitation test may throw light on this problem.

AGITATION OR SHAKING

The need of shaking or agitation of the ingredients of our test was believed to be due largely to the employment of a concentrated medium. Since the motion of the particles in such a medium is probably slow, it was believed that agitation hastened the interreaction between the particles. Of interest, however, was our finding that, while shaking invariably intensified precipitation in our test, the same did not hold true under modified conditions. Thus, when standard antigen dilution was employed with acidified or alkalinized serum, the effect of shaking was not at all constant. In certain proportions of the antigen dilution and modified serum, shaking intensified precipitation, while in other proportions shaking dissolved the precipitates which had been formed. The rôle of shaking will undoubtedly be understood when the other factors governing our test have been explained.

We have noted in the literature that certain workers tend to minimize the importance of shaking the ingredients in our test, claiming that similar results are obtained without shaking. It is true that the strongly positive sera do not require shaking, the precipitation reactions taking place within a few seconds after mixing serum with antigen dilution. In the case of weaker sera, however, our experience indicates that thorough agitation for several minutes is of the utmost importance.

INCREASING THE SENSITIVENESS OF PRECIPITATION REACTIONS

Our studies have shown that there are several ways by which the sensitiveness of precipitation reactions can be increased above that of the various procedures presented

in this volume. For practical reasons, fully discussed in the text, no attempt has been made to render these procedures as sensitive as possible. We have had occasion, however, in connection with special studies to attempt to increase the sensitiveness of precipitation reactions with isolated sera. A brief summary of the factors involved in increased sensitiveness is presented.

Increasing the age of the antigen-saline dilution. It has been shown that after mixing antigen with the required amount of salt solution, according to titration, there is a tendency for the antigen dilution to increase in sensitiveness on standing. This explains why 10 minutes are permitted to intervene between the dilution of antigen with salt solution and the employment of the antigen dilution with serum in the various procedures. Forty-five or 60 or more minutes, however, after diluting an antigen, cholesterin crystals begin to separate out and this renders the antigen dilution unfit for use.

The length of time an antigen dilution can stand without the appearance of cholesterin crystals varies somewhat with different antigens and can be readily determined by experiment. A given amount of antigen, titrated in the usual manner for use within 30 minutes after dilution, is mixed with salt solution according to titre. The solubility of the antigen precipitate is tested after 30 minutes' standing and then at 10 minute intervals by mixing 0.05 cc. quantities of antigen dilution with 1 cc. amounts of saline. After perhaps 60 minutes, cholesterin crystals begin to appear. In this case, the use of the antigen dilution with serum between 30 and 50 minutes after mixing the antigen with saline would be likely to give more sensitive reactions than between 10 and 30 minutes, as employed in the author's procedures, and at the same time cholesterin crystals would be avoided.

Decreasing the amount of antigen dilution in proportion to serum. Since it has been shown that with the decrease in the number of reacting substances in sera there must be a corresponding decrease in the quantity of antigen dilution in order to bring about complete precipitation, it is evident that the sensitiveness of reactions can be increased by the use of relatively smaller amounts of antigen dilution. For example, a proportion of serum to antigen dilution of 20:1 or 30:1 would give more sensitive reactions than 12:1 or 15:1.

Prolonging the heating periods of serum. Our studies have shown an apparent increase in sensitiveness of precipitation reactions after heating sera 1 hour at 56°C. compared with 30 minutes' heating at 56°C.

Increasing the length of the shaking period. Our studies have further shown that between 4 and 5 minutes' shaking of the tests results in slightly more sensitive reactions than 2 or 3 minutes' shaking.

STUDY OF INDIVIDUAL SERA FOR THE PRESENCE OF MINUTE AMOUNTS OF "REAGIN"

We have had occasion from time to time, in coöperation with clinicians, to carry on special investigations of sera for the presence of small amounts of syphilitic reacting substance. These sera gave negative reactions with all procedures. The clinician felt, however, that in these cases he had presumptive clinical evidence of syphilis. This led us to carry on special studies of the sera in question—making use of the ways, just discussed, of increasing the sensitiveness of precipitation reactions beyond that of our regular procedures. Obviously, it is the negative findings which have real value in such cases, since we know little regarding the specificity of positive findings.

Our procedure in such cases is briefly as follows:

The special antigen used in the presumptive test is employed. After an antigen dilution is prepared according

to the standard technic, it is permitted to stand 30 or more minutes before employing it with serum. The actual period can be readily determined by the method suggested in the previous discussion on factors governing sensitiveness of precipitation reactions.

The serum is heated for 60 minutes at 56°C. The serum tube should be properly corked during the heating period to avoid evaporation. One-hundredth cubic centimeter of the antigen dilution is measured into a tube in the usual manner. This is followed by 0.2 cc. of the heated serum. The mixture is shaken for 5 minutes in the shaking machine and then incubated for 15 minutes in the water bath. One-half cubic centimeter salt solution is added, mixed and the final result—positive or negative—read after 5 minutes' standing.

The control system discussed under the presumptive procedure applies also in this case.

This outline is presented with the hope that it may guide serologists in their attempt to aid clinicians in the diagnosis of special cases. As already indicated, a negative reaction is of special value in that it may help eliminate, in connection with clinical findings, the probability of syphilitic infection.

CLINICAL INVESTIGATIONS

One of the outstanding problems of the clinician is the correct interpretation of positive serologic reactions in syphilis. What does a so-called four plus reaction mean? Does it invariably indicate active syphilis or does it indicate active syphilis in some cases and possibly the development of immunity in other cases? Wile and Hasley insist that "In the presence of an intensive therapy, a positive test does not necessarily mean living spirochetes and potential syphilis any more than a positive tuberculin test in an individual who has had tuberculosis would indicate the presence of living tubercle bacilli." Many clinicians, on

the other hand, believe that the presence of a positive serologic reaction is a definite indication of active syphilis. According to this view a positive serologic reaction invariably means that the patient has syphilis, while, according to Wile's view, a positive reaction means that the patient has or has had syphilis.

When considering the large number of cases which become clinically cured by treatment—and apparently remain cured for many years—but which nevertheless continue to give positive serologic reactions, it is difficult to see how such reactions could always mean active syphilis. Unless we assume that it is possible to have active syphilis and at the same time be in good health and have healthy children, we are forced to agree with Wile that a positive serologic reaction does not necessarily mean the presence of active spirochetes.

Even if we accept the last view, however, there remain many problems associated with the clinical interpretation of serum reactions in syphilis. We recently had occasion to examine with our quantitative procedure the serum of an untreated patient with a tertiary lesion on the forehead. The results showed that the serum contained over 2000 reacting units. The number of reacting units usually found in active secondary syphilis is between 80 and 200. We thus have a condition in which the serum of a patient having a localized lesion contains many times the amount of "reagin" contained in the serum of a patient having generalized syphilis.

Obviously it is the clinician's field to ultimately establish the true interpretation of serologic reactions and, when attempting to obtain light on this problem, the author's various procedures, when correctly carried out by the serologist, should prove helpful. The clinician has for his guidance in the interpretation of clinical findings not merely a serologic test for syphilis, but a series of procedures

which can give him considerable information. Thus, a serum which gives a four plus reaction with the routine test will be positive with the presumptive procedure and will be found to contain a certain number of reacting substances by the quantitative method. In such a case, the presumptive procedure serves as a check on the findings of the routine test and the quantitative method makes it possible to follow the serologic effect of anti-syphilitic treatment quantitatively. If a serum gives a negative or doubtful reaction with the routine test, a positive reaction with the presumptive procedure may prove an indicator as to continuation of treatment or may guide in the diagnosis of obscure cases. If a serum is negative with both the routine test and presumptive procedure, it may be further investigated for the presence of "reagin" according to the outline presented in this Chapter. The correlation, by clinicians, of the results obtained with these different procedures in a large number of cases should ultimately lead to a better understanding of the interpretation of serologic reactions in syphilis.

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INDEX

A

- Absorption of syphilitic reacting substances, 63, 206.
- Acid, in antigen, 111.
 - in glassware, 109, 130.
 - in salt solution, 109.
 - in serum, 111, 212.
 - use for precipitating spinal fluid globulins, 153.
- Active syphilis, the test in, 174.
- Age, of antigen (keeping quality), 133.
 - of antigen dilution and sensitive-ness, 51, 204, 214.
 - of serum (and contamination), 86, 88, 212.
- Agglutination (clumping) of pre-cipitates, 4, 39.
 - effect of concentration on, 4, 35, 39.
 - effect of incubation on, 39.
- Agglutinoscope, for Meinicke reac-tion, 10.
 - for Sachs-Georgi reaction, 11.
 - for Sigma reaction, 12.
 - Kuhn-Voite, 11.
- Agitation of tests, *see* Shaking.
 - importance of, 32, 34, 90, 213.
 - in standard procedures, 95, 140.
 - various periods of, 91.
- Alcohol extraction of beef heart, 25, 76, 133.
 - antigenic value of direct, 26.
 - antigenic value of secondary, 117, 120, 210.
 - in standard antigen preparation, 76.
 - temperature of, 76.
 - uniformity of, 77.
- Alcohol for beef heart extraction, 133.
- Alkali, in antigen, 113.
 - in glassware, 109, 130.
 - in salt solution, 112.
 - in serum, 112, 212.
- Ammonium sulphate, chemical pur-ity of, 154.
 - precipitation with antigen dilu-tion, 153, 156.
 - saturated solution for precipita-tion of spinal fluid globulins, 154.
 - use in special titration of antigen, 156.
- Anderson, 167.
- Anomalous reactions, 64, 183.
- Anticomplementary sera, in Kahn test, 89.
 - in Wassermann test, 89.
- Antigen, acetone insoluble, 11, 20, 116, 117, 120, 198.
 - acid added to, 111.
 - alcoholic extract, "crude," 26, 28, 119.
 - alcoholic extract, "refined" or "purified," 26, 28, 119.
 - alkali added to, 113.
 - an especially sensitive, Chapter XIV.
 - balsam toltutanum added to, 14.
 - Bordet, 15.
 - Bordet and Ruehlens, 12.
 - chemical studies of, 205, 210.
 - cholesterin-free, 77.
 - cholesterinization of, 77.
 - cholesterinization of standard, 133.
 - concentrated, 25.

- different antigens in routine test, 115, 209.
 dilution of, 49.
 Kolmer, 116, 198, 209.
 lecithin suspension as, 7.
 lipid content increased with cholesterolin, 20.
 Meinicke, 9, 20.
 Neumann and Gager, 20, 21, 23, 25, 26, 28.
 Noguchi, 20, 21, 23, 25, 26, 28, 116, 198, 209.
 preparation of special, 160.
 preparation of standard, 131.
 quantitative demands of, 5, 54.
 resin of benzoin added to, 15, 199.
 Sachs-Georgi, 10, 20.
 sensitiveness and uniformity of, Chapter VII.
 use of undiluted, 20, 25.
 wet heart muscle, 10, 25, 119.
see under standard antigen.
see under special antigen.
- Antigen dilution, component responsible for specific precipitation, 99.
 effect of age or standing, 51, 145, 204, 214.
 excessive amount of, 54.
 frequent mixing during period of pipetting, 145.
 instability of precipitate in, 42, 46, 201.
 minimum amount of salt solution, 25, 26, 44, 46.
 physical character of, 48, 203, 205.
 pipettes kept in salt solution during period of pipetting, 145.
 pipetting of, 140.
 preparation of special, 163.
 preparation of standard, 139.
 proportions of antigen and salt solution, 44.
 uniformity of, 49.
- Antigen preparation, alcohol extraction, 76.
 cholesterinization, 77.
 ether extraction, 68.
 special antigen, 160.
 standard antigen, 131.
 uniformity in, 68.
- Antigen-saline precipitate, instability of, 46, 201.
 solubility of, 42, 137.
- Apparatus, standard, 129.
- Application, of presumptive procedure, 191, 194.
 of procedure for study of special cases, 216, 218.
 of quantitative procedure, 187.
 of routine test, 180.
 of spinal fluid procedure, 190.
- ### B
- Babonneix, 167.
 Bacto beef heart, 132.
 Bacterial contamination of serum, in Kahn test, 86, 88.
 in Meinicke reaction, 19.
 in Sachs-Georgi reaction, 19.
 in Wassermann test, 86, 88.
- Balsam tolutanum, in antigen, 14.
- Beef heart muscle, dry, 25.
 powdered, 25, 131.
 relation to syphilis, 6, 197, 211.
 wet, 25.
- Bent, 167.
- Blood, obtaining for test, 135.
- Blood transfusion cases, the test in, 4.
- Bordet, 12, 15.
- Boucher, 167.
- Bruck, 7, 13, 200.
- ### C
- Calf heart muscle for antigen preparation, 11.

- Cerebrospinal syphilis, comparative findings of Kahn and Wassermann, 189.
routine test in, 174.
spinal fluid procedure in, 188.
- Chancre fluid, reaction with, 147, 180.
- Chemical studies, importance of, 205, 213.
- Choay, 167.
- Cholesterin, insolubility of, 103, 117, 120, 204, 214.
in special antigen, 161.
in standard antigen, 133.
necessity for pure product, 133.
relation to sensitiveness, 79.
rôle of, 79.
technic of adding to extract, 133.
- Chylous serum, 135.
- Clinical application of presumptive procedure, in obscure cases, 193, 194.
interpretation of results, 191.
in treated cases, 192, 194.
- Clinical application of quantitative procedure, study of different modes of treatment, 186.
study of treated cases, 185.
- Clinical application of procedure for study of special cases, 216, 218.
value of negative findings, 215.
- Clinical application of routine test, in diagnosis, 172.
in treated cases, 181.
- Clinical application of spinal fluid procedure, in neurosyphilis, 159, 188.
in treated cases, 159, 188.
- Clinical investigations, 216.
- Clumping (agglutination) of precipitates, effect of concentration on, 4, 35, 39.
effect of incubation on, 39.
- Coca, 65.
- Complement destruction in heated sera, 81.
- Complement fixation test (Wassermann), comparative findings with Kahn test, 167, 181.
indirect nature of, 58, 66, 197.
- Concentration, a governing principle of precipitation in syphilis, 3, 19, 198.
and agglutination of precipitates, 4, 35, 39.
and immediate reactions, 3, 35.
and elimination of incubation, 31.
and sensitiveness, 4, 44.
applied to antigen, 25, 198.
applied to ingredients of test, 25.
limitations of principle of, 199.
of reacting substances of spinal fluids, 154.
rôle of, Chapter IV.
versus dilution in three precipitation tests (table), 41.
- Congenital syphilis, the test in, 177.
- Contamination of sera, in Kahn reaction, 86, 88.
in Meinicke reaction, 19.
in Sachs-Georgi reaction, 19.
in Wassermann reaction, 86, 88.
- Control, antigen, 142.
negative serum, 143.
positive serum, 143.
serum, 142.
- Control system, for presumptive procedure, 164.
for routine test, 142.
for spinal fluid procedure, 155.
- Cork stoppers, 133.
- Cornwall, 15.
- Correct diagnosis of syphilis, importance of, 194.
- Correlation of various procedures, 218, *see* Summary Chart.
- Crystallization of cholesterin from antigen dilution, 103, 120, 204, 214.

Curve showing relation between reacting units and serum dilution, 62.

D

Delaying of precipitation, by dilution, 3, 23, 37.

by excessive antigen dilution, 54.
by excessive serum, 64.

Detweiler, 167.

Diagnosis of syphilis, limitations of serum reactions in, 6.

Dilution, delaying effect on precipitation, 3, 23, 37.

in relation to *B. typhosus* agglutination, 200.

of antigen for tests necessary, 25.

of serum for quantitative procedure, 60.

of standard antigen, 139.

of special antigen, 163.

versus concentration in three precipitation tests, 40.

Dold, 12.

Dreyer, 11.

Dujarric de la Rivière, 15, 199.

Dulaney, 167.

E

Elimination of incubation period, 31.

Elliott, 167.

Emergency cases, necessity for rapid test in, 4.

Erlandson, 210.

Ether, over-extraction with, 69, 72, 121.

preliminary extraction of beef heart with, 68.

Ether extraction, antigens with various degrees of, 73, 122.

limitation of, 72, 121.

necessity for, 68.

standardized technic for, 76, 132.

Ether extractives, addition to antigens of, 71.

in relation to sensitiveness, 68, 208.

standardization of antigens with, 71, 208.

Evaporation, of antigen dilution, 146.

of serum-antigen mixtures, 105.

Extracts, of animal tissue, 3.

of powdered beef heart, 25.

of syphilitic liver, 7.

of wet beef heart muscle, 10, 25, 119.

"crude" alcoholic, 26, 28, 119.

"refined" alcoholic, 26, 28, 119.

F

Filter paper used in antigen preparation, 129, 133.

Fisher, 167.

Flask for standard antigen preparation, 129.

Flocculation tests by different authors, *see* Precipitation tests.

Fluctuations, inherent in beef heart extracts, 5, 69.

in results of routine test, 108.

in sensitiveness of antigens, 30, 70, 207.

Fluids from syphilitic lesions, reactions with, 147, 180, 193.

Formula expressing reaction units of serum, 62.

Fox, 167.

G

Gager, 20, 21, 23, 25, 26, 28, 201, 210.

Gallerand, 15, 199.

Georgi, 10, 12, 19, 207.

Glassware used in test, Chapter XV.
neutrality of, 130.

standard, 129.

washing of, 129.

Globulin concentration, in spinal fluids, 154.

in serum, 212.

Grant, 167.

Grün, 14.

H

Hasley, 216.

Havens, 167.

Hecht, 8, 13.

Heating sera, effect on results of Kahn, 80.

effect on results of Wassermann, 80.

Hemolysis of blood, effect upon test, 135.

causes of, 135.

Hereditary syphilis, the test in, 177.

Herrold, 153, 167, 212.

Hidaka, 7.

H-ion concentration of sera, effect on reaction, 81.

Holmes, 167.

Hopkins, 167.

Horse heart for antigen preparation, 9.

Human heart for antigen preparation, 13.

I

Ide, 167.

Inactivation of sera, necessity for, 80.

period at 56° C., 82, 135.

re-inactivation, 86, 135.

Incubation, advantage of 15 minute period in routine test, 141.

and bacterial contamination, 19.

effect on test, 104, 140.

elimination from routine test, 31.

non-specific factors accompanying, 197.

overcomes delaying effect of dilution, 37.

in Meinicke reaction, 9.

in Sachs-Georgi reaction, 10.

in Sigma reaction, 11.

Ingredients of test, Chapter XVI. antigen, 131.

physiologic salt solution, 135.

serum, 134.

Inhibition of precipitation, by dilution, 3, 23, 37.

by excessive antigen dilution, 54.

by insufficient heating of sera, 81.

Ishii, 167.

Instability of antigen dilution, Chapter V.

a governing principle of precipitation in syphilis, 4, 42, 201.

Interpretation of results, in nomenclature suggested by League of Nations' Health Committee, 142.

of antigen titrations, 137.

of presumptive procedure, 164.

of quantitative procedure, 152.

of routine test, 143.

of serologic reactions, 197, 216, 218.

of spinal fluid procedure, 155.

J

Jacobstahl, 7.

Johnson, 67.

K

Keim, 154, 167, 172, 188.

Kendrick, 88, 167, 200, 211.

Klausner, 7.

Kolmer, 66, 116, 167, 198, 209.

Kuhn-Voite agglutinoscope, 11.

L

Laboratory apparatus for test, 129.

Latent syphilis, the test in, 174.

L'Esperance, 65.

Lecithin suspensions as antigen, 7.

Levin, 167.

Lipoidbindungsreaktion, 10.

Litterer, 167.

Lues, *see* Syphilis.

M

Madsen, 142.

Malcolm, 167.

Mannheims, 167.

Marshall, 81.

- McClean, 167.
 Meier, 7.
 Meinicke, 8, 9, 10, 12, 14, 19, 199, 200.
 Michaelis, 7.
 Minimum amount of salt solution,
 indicated by antigen titration,
 51, 136.
 used in standard antigen dilution,
 137.
- N
- Nedley, 167.
 Neisser, 7.
 Neumann, 20, 21, 23, 25, 26, 28,
 201, 210.
 Neurosyphilis, routine test in, 174.
 spinal fluid procedure in, 159.
 Neutrality of glassware, 130.
 Noguchi, 20, 21, 23, 25, 26, 28, 116,
 198, 209.
 Nomenclature of results, of pre-
 sumptive procedure, 164.
 of quantitative procedure, 152.
 of routine test, 143.
 of spinal fluid procedure, 155.
 suggested by League of Nations'
 Health Committee, 142.
 Novy, 212.
- O
- Opalescence, of antigen-saline mix-
 ture, 26, 42, 201.
 of negative reactions, 141.
 Osmond, 167.
 Ottenberg, 66.
- P
- Physical character of antigen dilu-
 tion, changes in, 203.
 relation to sensitiveness, 48, 102,
 205.
 unstable antigen dilution pre-
 cipitate, 46.
 Pipettes, for antigen dilution, 129.
 for serum, 129.
 marking of, 145.
 Poehlmann, 11.
 Porges, 7.
 Powdered beef heart, alcohol ex-
 traction of, 76, 133.
 Bacto beef heart, 132.
 ether extraction of, 76, 132.
 importance of, 19, 20.
 preparation of, 131.
 uniformity of, 131, 132.
 use in standard antigen prepara-
 tion, 131.
 Precipitation system proposed by
 Kahn, *see* Summary Chart.
 Precipitation tests proposed by
 different authors, Chapter II.
 Bruck, 13.
 Bruck and Hidaka, 7.
 Dold, 12.
 Dreyer and Ward, 11.
 Dujarric de la Rivière and
 Gallerand, 15.
 Hecht, 13.
 Jacobstahl, 7.
 Klausner, 7.
 Meinicke, 8, 9, 14.
 Meinicke and Grun, 14.
 Michaelis, 7.
 Porges and Meier, 7.
 Sachs and Georgi, 10.
 Vernes, 15.
 Wang, 13.
 Preliminary procedure of author's
 test, 28, 30.
 antigen used in, 29, 30.
 comparative findings with Was-
 sermann, 30.
 incubation period, 29, 30.
 interpretation of results, 29.
 preparation of antigen-saline
 dilution, 29.
 reading of results, 29.
 value of, 31.
 Presumptive procedure, antigen
 preparation for, 160.
 clinical application of, 191, 194,
 218.

- control system, 164.
- development of, 121.
- interpretation of results, 164.
- reading of results, 163.
- specificity of, 192.
- technic of procedure, 163.
- titration of antigen, 161.
- use as check on routine test, 191.
- Primary syphilis, comparative findings of Wassermann and Kahn, 174.
- Problems to be solved, *see* Chapter XXVIII.
- Procedures, preliminary, 28.
 - presumptive, 160.
 - procedure for study of special cases, 215.
 - quantitative, 148.
 - routine test, 139.
 - spinal fluid procedure, 153.
- Proportions of serum and antigen dilution, employed in routine test, 33, 57.
 - importance of correct, 4, 33, 54.
 - when using non-standard quantities, 147.
- Public Health Laboratory, one hundred thousand examinations in a, 167.
 - representative day's serological tests in a, 169.

Q

- Quantitative procedure, based on unit system, 148, 185.
 - in study of new modes of treatment, 186.
 - in treated cases, 185, 218.
 - serum dilutions for, 150.
 - technic of, 150.
 - unit reaction of, 149.
- Quantitative relation between serum and antigen, Chapter VI.
 - importance of correct, 5, 33, 54, 205.

R

- Rack, standard, 129.
- Reacting substances (syphilitic),
 - absorption of, 63.
 - determination of, 58.
 - importance of determining, 65.
 - minute amounts of, 215.
 - quantitative relation with antigen substances, 4, 33, 54.
- Reacting units, determination by quantitative procedure, 149.
 - measurement by precipitation test, 59.
 - measurement by Wassermann test, 58.
 - reduction during treatment, 186.
- Sigma, 12.
- Reactions (precipitation), anomalous, 64, 183.
 - increasing sensitiveness of, 213.
 - in presumptive procedure—interpretation, 164.
 - in procedure for study of special cases—interpretation, 216.
 - in quantitative procedure—interpretation, 152.
 - in routine test—interpretation, 143.
 - in spinal fluid procedure—interpretation, 155.
 - reading of, 141.
 - value of immediate, 4.
- Reading results, 141.
 - importance of check reading, 142.
 - optimum lighting conditions for, 141.
- Reagin, detection of minute amounts, 215.
- Re-inactivation of sera, importance of, 86.
- Requirements for a practical test,
 - concentration of ingredients, 35.
 - factors affecting serum, 80.
 - instability of antigen dilution, 42.

- quantitative serum-antigen relation, 54.
 sensitiveness of antigen, 68.
 shaking (agitation), 90.
 uniformity of antigen, 68.
 Research, application of author's system to, 198.
 Resinous matter, in antigens, 15.
 Ring modification of Kahn test, 153.
 Rockstraw, 167.
 Rondoni, 49.
 Rothberg, 167.
 Routine test with serum, Chapter XVIII.
 agitation (shaking), period of, 140.
 apparatus, 129.
 control system, 142.
 correlation with other procedures, 218.
 diagnostic value, 180.
 effect of incubation, 140.
 final result, 144.
 inactivation of sera, 135, 140.
 in a public health laboratory, 169.
 in cases undergoing treatment, 181.
 in non-syphilitic cases, 179.
 interpretation of results, 143.
 in various stages of syphilis, 180.
 large numbers of specimens, 145.
 performance of test, 140.
 preparation of antigen dilution 139.
 preparation of serum, 134.
 proportions of serum and antigen [dilution, 140.
 reading results, 141.
 sensitiveness compared with Wassermann test, 168, 180.
 standard glassware, 129.
 use of non-standard quantities, 147.
 Rubber stopper, disadvantages of, 133, 200.
 Ruehlens, 12.
- S
- Sachs, 10, 12, 19, 49, 205, 207.
 Salt solution, experiments with half normal, 37.
 experiments with 2 per cent, 37.
 Salt solution (physiologic), acid in, 109.
 alkali in, 112.
 chemical purity of, 135.
 delaying effect on precipitation, 35, 37.
 preparation, 135.
 standardization of antigens with, 51.
 sterility of, 135.
 use of minimum amount in antigen dilution, 25, 26, 44, 46, 202.
 Sanderson, 167.
 Secondary syphilis, comparative findings of Wassermann and Kahn, 174.
 Sensitiveness of reactions, and age of antigen dilution, 51.
 and alcohol extraction of antigen, 68, 76.
 and cholesterinization of antigen, 77.
 and concentrated antigen, 19, 25.
 and ether extraction of beef heart, 68, 121.
 and excessive antigen dilution, 54.
 and excessive serum, 64.
 and instability of antigen dilution, 44, 46.
 and physical character of antigen dilution, 48.
 and specificity, 58.
 effect of concentration on, 4, 44.
 effect of dilution on, 35.
 effect of heating serum on, 80, 86, 215.
 effect of incubation on, 104.
 effect of shaking on, 90.
 methods of increasing, 213.
 serum-antigen proportions, 5, 54, 215.

- Serum, acid added to, 111, 212.
alkali added to, 112, 212.
anticomplementary, 89.
chylous, 135.
control in presumptive procedure, 164.
control in routine test, 142.
digestion of, 212.
dilutions for quantitative procedure, 150.
effect of age on, 86, 88.
effect of contamination on, 86, 88.
effect of H-ion concentration, 81.
factors affecting, Chapter VIII.
globulin precipitation with ammonium sulphate, 212.
heated versus unheated, 80, 211.
maximum dilution giving reaction in quantitative procedure, 149.
necessity for inactivation of, 80.
period of inactivation of, 82.
preparation for test, 134.
re-heating of, 86.
re-inactivation of, 86.
use of excessive amount of, 64.
use of undiluted, 25, 26.
- Shaking (agitation) of tests, and negative reactions, 90.
and strongly positive reactions, 90, 213.
and weakly positive reactions, 91, 213.
effect of prolonged, 95.
importance of, 32, 34, 90, 213, 215.
in acid and alkali experiments, 109, 112, 213.
period in presumptive procedure, 163.
period in quantitative procedure, 150.
period in routine test, 95, 140.
period in spinal fluid procedure, 155.
period in procedure for study of special cases, 216.
various periods of, 91.
- Shaking machine, description, Plate III.
desirability of, 129, 146.
Sigma reaction, 11.
Smith, 167.
Solubility of antigen dilution precipitate, 31, 42, 137.
Special antigen, preparation of, 160.
sensitivity of, 121.
use in presumptive procedure, 160.
use in quantitative procedure, 149.
use in spinal fluid procedure, 157.
use in study of special sera, 215.
- Special antigen dilution, preparation of, 163.
- Specimens (blood), collection of, 135.
examination of large numbers of, 145.
hemolysis of, 135.
unsatisfactory, 88.
- Spinal fluid procedure, Chapter XX.
concentration of spinal fluid globulins, 154.
control system, 155.
clinical application, 159.
interpretation of results, 155.
performance of test, 155.
preparation of special antigen dilution, 163.
preparation of spinal fluid, 154.
preparation of standard antigen dilution, 155.
titration of special antigen, 159.
titration of standard antigen, 156.
- Stable antigen-saline precipitate, 4.
- Standard antigen, aim in preparation of, 121.
alcohol extraction, 133.
cholesterinization, 133.
dilution for test, 139.
ether extraction, 132.
keeping quality of, 133.
preparation of, 131.
titration for routine test, 136.

- titration for spinal fluid procedure, 156.
- Standard antigen dilution, effect of age on sensitiveness, 51.
- pipetting of, 140.
- preparation of, 139.
- prevention of evaporation of, 146.
- separation of cholesterin from, 120.
- standard tubes for, 129.
- Standardization of antigen, for presumptive procedure, 161.
- for routine test, 136.
- for spinal fluid procedure, 156, 159.
- with negative serum, 137.
- with salt solution, 51, 137.
- Standard rack, 129.
- Standard test tubes, for preparation of antigen dilution, 129.
- for use in test, 129.
- Stigma associated with syphilis, 194.
- Stock alcoholic antigen, 133.
- Strumia, 167.
- Summary chart, *faces* 218.
- Syphilis, cerebrospinal, 174.
- cutaneous involvement, 175.
- general paresis, 176.
- hereditary (congenital), 177, 179.
- latent, 174.
- obscure manifestations of, 194.
- of osseous system, 175.
- of visceral system, 175.
- primary, 173.
- secondary, 174.
- stigma associated with, 194.
- tabes dorsalis, 177.
- tertiary, 175.
- treated cases of, 184.
- T
- Talc, use in spinal fluid globulin precipitation, 154.
- Taylor, 167.
- Technic, antigen preparation, 131.
- antigen titration, 136.
- dilution of antigen, 139.
- powdered beef heart preparation, 131.
- preparation of glassware, 129.
- presumptive procedure, 163.
- procedure for study of special cases, 215.
- quantitative procedure, 150.
- routine test, 139.
- spinal fluid procedure, 154.
- Temperature, of alcohol extraction in antigen preparation, 76.
- of ether extraction in antigen preparation, 72.
- of inactivation, 82.
- Tertiary syphilis, the test in, 174.
- Thermolability of reacting substances, 211.
- Thermostability of reacting substances, 211.
- Tinfoil, for covering cork stoppers, 133.
- Titration, of antigens for spinal fluid procedure, 156, 159.
- of special antigen for presumptive procedure, 161.
- of standard antigen for routine test, 136.
- purpose of, 136.
- Todd, 167.
- Treated cases, comparative findings of Kahn and Wassermann in, 181.
- presumptive procedure in, 192, 194.
- quantitative procedure in, 187.
- routine test in, 181.
- spinal fluid procedure in, 159.
- Tubes, for tests, 129.
- for diluting antigen, 49, 129.

U

Ultramicroscope, Jacobstahl method, 7.

Uniformity, in antigen preparation, 5, 68, 206.

in dilution of antigen, 32, 49.

Unit reaction, derivation of, 59, 148.

in quantitative procedure, 149.

interpretation of, 60.

V

Variation of results, in hands of different workers, 5.

with different precipitation tests, 5.

Vernes reaction, 15.

W

Wang, 13.

Ward, 11.

Wassermann, 7, 20.

Wassermann test, 7, 20, 58, 66.

inactivation of sera for, 80.

results compared with routine test, 167, 181.

results compared with spinal fluid procedure, 189.

technic used in comparative studies, 168.

Wile, 66, 167, 216, 217.

Wilson, 167.

Y

Yagle, 167.

Young, 167.

Z

Zone phenomenon, 111, 114, 212.

Zones, reacting, 65.

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